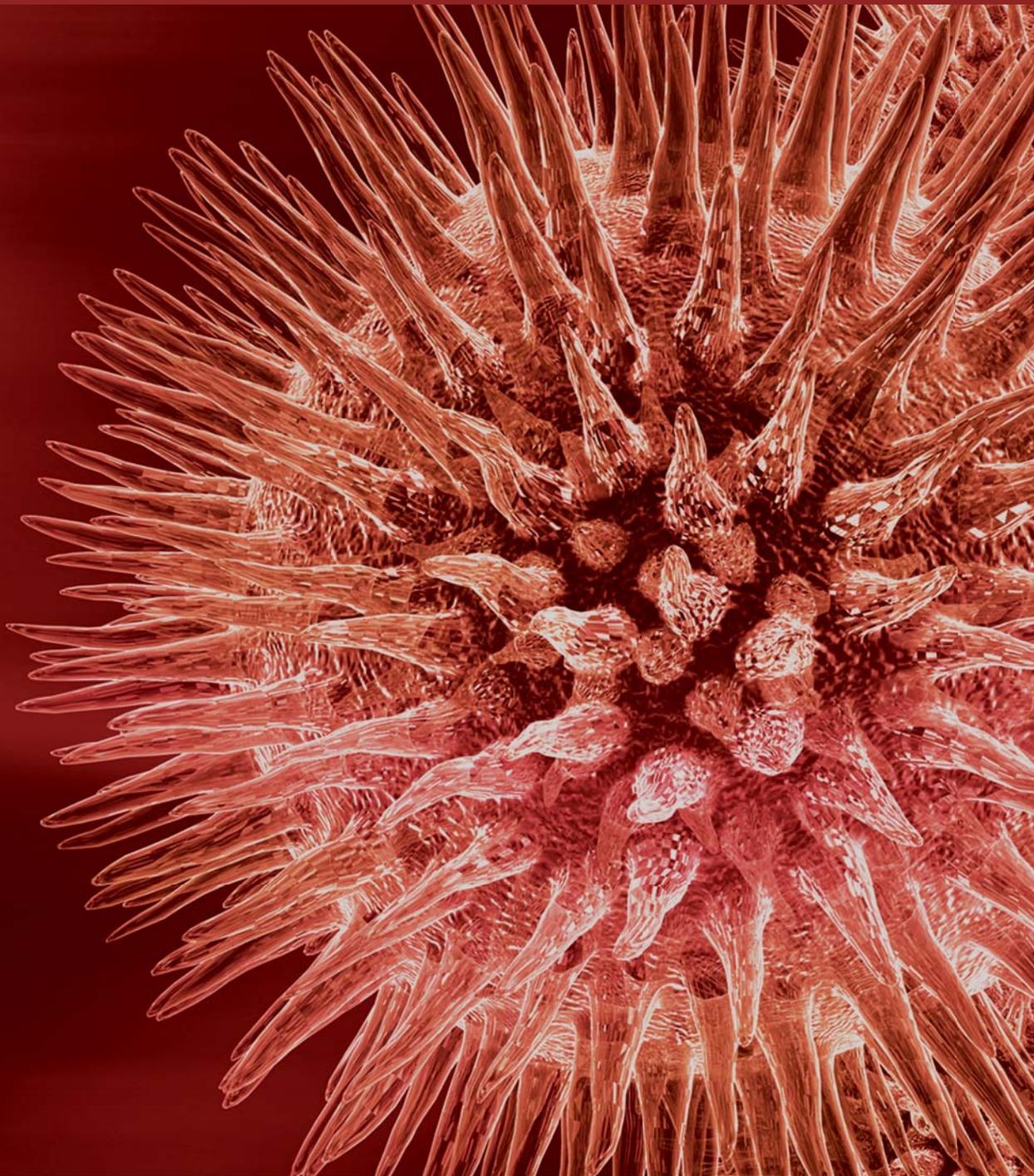


Protein Acetylation and the Physiological Role of HDACs

Guest Editors: Patrick Matthias, Christian Seiser, and Minoru Yoshida





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Editorial

Protein Acetylation and the Physiological Role of HDACs

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It was in 1964 that V. G. Allfrey and colleagues first reported the isolation of acetylated histones and speculated—well ahead of their time—about their possible role in RNA synthesis [1]. About ten years later, the nucleosome was defined as the basic unit of chromatin [2] and soon thereafter DNase I hypersensitivity analysis of isolated HeLa cells nuclei demonstrated that histone acetylation impacts on chromatin structure [3].

Since then, the importance of acetylation for the regulation of chromatin and gene activity has been demonstrated by numerous studies and this posttranslational modification is now rivaling phosphorylation in its importance. The field benefitted enormously from the early identification of molecules, such as Trichostatin A, which inhibit the enzymes removing acetyl groups—histone deacetylases (HDACs)—and thus lead to hyperacetylation of histones and chromatin [4]. These HDAC inhibitors (HDACis) turned out to have very interesting biological effects, such as induction of differentiation in cellular model systems. In addition, it had been realized that HDACis show antiproliferative potential when applied to cultures of transformed cells [5] and this sparked an enormous interest in their potential use for therapeutic purposes. A variety of substances, coming from natural or synthetic sources, have been tested in cancer models, and also in other pathologies, such as neurodegeneration, autoimmunity, or inflammation: in many cases target they were found to be beneficial. These inhibitors usually all or most of the HDACs—there are eleven of them—and therefore it is not clear yet which HDAC(s) are implicated in which pathology. The last ten years have witnessed a wealth of clinical trials, primarily in cancer, and also more recently in other settings, and today two inhibitors—SAHA and romidepsin—have

been approved for clinical treatment of cutaneous T cell lymphoma. In parallel to this, genetic analysis of HDACs has progressed, in particular in the mouse, where all HDACs have now been ablated. This analysis revealed that some HDACs, such as HDAC1 or 3, are essential genes [6, 7], while others are dispensable for development, but show specific phenotypes when ablated, for example, organism-wide increased tubulin acetylation in the case of HDAC6 knockout mice [8]. In addition, conditional alleles of these and other HDACs have been generated allowing to test their function in specific organs or in combinations, by using appropriate Cre-expressing mice lines. These studies identified important roles for HDACs, for example, in the nervous system, in the heart, or in lymphocytes [9–11].

This special issue deals with “protein acetylation and the physiological role of HDACs.” As should be evident from the important short introduction above, this is an exciting topic which has implications for basic research and a demonstrated increasing medical relevance.

Several reviews address the general role or regulation of HDACs (T. Hayakawa and J. I. Nakayama; C. Segre and S. M. Chiocca; A. Peserico and C. Simone). A number of reviews cover our recent understanding of the role of HDACs in cancer and various models are discussed, such as—among others—leukemia (C. Biagi et al.; L. Bagella and M. Federico), pancreatic cancer (A. Ouaiissi et al.; C. Bevan and D. Lavery), breast cancer (A. Linares et al.), or the link between autophagy, apoptosis, and HDAC inhibition in cancer cells (H. Rikiishi). Also, several reviews address important aspects of HDAC function on nonhistone proteins (e.g., on interferon regulatory factor, A. Masumi) and in particular their role in the cytoplasm (S. Khochbin et al.; W.-M. Yang and

Y.-L. Yao; C. Creppe and M. Buschbeck). The importance of HDACs on cardiac development and function or in hypoxia is also addressed (H. Kook and H. J. Kee; N. Sang and S. Chen) and a number of additional topics are touched upon by dedicated reviews or a few primary data papers.

In summary, this special issue gives an excellent overview of the current status of research on HDACs and should be a valuable source of reference material for students or researchers.

Patrick Matthias
Christian Seiser
Minoru Yoshida

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Review Article

Sirtuins: Molecular Traffic Lights in the Crossroad of Oxidative Stress, Chromatin Remodeling, and Transcription

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Transcription is regulated by acetylation/deacetylation reactions of histone and nonhistone proteins mediated by enzymes called KATs and HDACs, respectively. As a major mechanism of transcriptional regulation, protein acetylation is a key controller of physiological processes such as cell cycle, DNA damage response, metabolism, apoptosis, and autophagy. The deacetylase activity of class III histone deacetylases or sirtuins depends on the presence of NAD⁺ (nicotinamide adenine dinucleotide), and therefore, their function is closely linked to cellular energy consumption. This activity of sirtuins connects the modulation of chromatin dynamics and transcriptional regulation under oxidative stress to cellular lifespan, glucose homeostasis, inflammation, and multiple aging-related diseases including cancer. Here we provide an overview of the recent developments in relation to the diverse biological activities associated with sirtuin enzymes and stress responsive transcription factors, DNA damage, and oxidative stress and relate the involvement of sirtuins in the regulation of these processes to oncogenesis. Since the majority of the molecular mechanisms implicated in these pathways have been described for Sirt1, this sirtuin family member is more extensively presented in this paper.

1. Introduction

Acetylation is the addition of an acetyl group at the ϵ -amino group of the lysine residues present within histone and nonhistone proteins and is one of the most extensively studied posttranslational modifications [1]. Acetylation is mediated by enzymes called histone acetyl transferases (HATs), but since a large number of nonhistone proteins are targeted by HATs, these enzymes are also called KATs (K-acetyltransferases) [2]. The removal of the acetyl group is regulated by the activity of histone deacetylases (HDACs). Acetylation of histone tails decreases their net positive charge [3], thereby reducing the chromatin-binding affinity to DNA, which then becomes more accessible to the transcription initiation complexes and the RNA polymerase [4, 5]. The pattern of the N-terminal histone posttranslational

modifications mediating transcriptional events is called “histone code” [6].

Transcriptional regulation of gene expression is a complex process involving several posttranslational modifications of histone and nonhistone proteins. The balance between reversible modifications such as acetylation, phosphorylation, methylation, ubiquitination, propionylation, butyrylation, carbonylation, and ADP ribosylation, occurring within specific chromatin domains, controls the expression or silencing of a diverse set of genes [7]. Enzymes regulating the equilibrium of these modifications maintain the chromatin organization and structure, thus fine-tuning the expression of individual genes. Acetylation of the protruding histone tails is generally associated with activation of gene expression whereas deacetylation is linked to inhibition of gene expression [8]. HDACs exert their repressive function

on transcription either by condensing the chromatin or as components of large multiprotein complexes, by recruiting inhibitory factors to regulatory DNA elements within gene promoter regions [9]. Transcriptional regulation exerted by HDACs determines vital cellular processes including cell cycle progression, apoptosis, autophagy, response to diverse types of stress, differentiation, and development [10]. Alterations in HDACs-mediated signaling due to overexpression or hyperactivity of these enzymes can lead to disturbed homeostasis and, hence to pathological conditions [11] including systemic autoimmune [12], Huntington's [13], neurodegenerative [14], respiratory [15], and cardiovascular diseases [16], inflammation [17], diabetes [18], cardiac hypertrophy [19, 20], cancer [21], and conditions such as ageing [22, 23].

Eighteen eukaryotic HDACs, bearing a common well-conserved catalytic deacetylase domain, have been identified so far and classified into four classes: I, II, III, and IV [24]. HDAC1, HDAC2, HDAC3, and HDAC8 are members of the class I HDACs similar to *Saccharomyces cerevisiae* reduced potassium deficiency 3 (Rpd3) deacetylase. They are usually localised in the nucleus and form large multiprotein complexes which confer to these enzymes strict specificity for particular acetylation sites [25]. Class I HDACs can be further divided into HDAC1/HDAC2 and HDAC3 subclasses. Class II members (HDAC 4, 5, 6, 7, 9, and 10) are homologous to the yeast Hda1 deacetylase and can be further subdivided into class IIa (HDAC4, 5, 7 and 9) and IIb (HDAC6 and 10) [26, 27]. Class II HDACs are localized in both the nucleus and the cytoplasm to target histone and nonhistone proteins. HDAC11-related enzymes are considered to form a separate type of HDACs the class IV [28].

The class III HDACs or sirtuins consists of seven members (Sirt1–7) homologous to the yeast HDAC silent information regulator 2 (Sir2) (Figure 1). The common characteristic of this class is that they are nicotinamide adenine dinucleotide (NAD⁺-) dependent enzymes [29, 30]. The requirement of the NAD⁺ cofactor and the mitochondrial localisation of some sirtuin family members imply a role of this class of deacetylases in the regulation of the metabolic homeostasis and suggest that histones are not their primary targets. Sirtuins show significant sequence and functional differences from other classes of HDACs in that they carry out deacetylation via a two-step reaction that consumes NAD⁺ and releases nicotinamide (NAM), O-acetyl-ADP-ribose (AADPR), and the deacetylated substrate [31]. Sirtuins, although relatively similar to each other have divergent biological functions due to distinct cell-type-specific subcellular localisation of each member of the family [31]. In particular, Sirt1 is located in both the nucleus and the cytoplasm, Sirt2 in the cytoplasm, Sirt3, 4, and 5 are mitochondrial, and Sirt6 and 7 are exclusively nuclear [32, 33]. Apart from intracellular localization, Sirt1, 3, and 5 differ from Sirt2, 4, and 6 in the type of reaction they catalyse. Sirt1, 3, and 5 are NAD⁺-dependent deacetylases catalyzing the deacetylation of histones and nonhistone proteins, whereas Sirt6 is a NAD⁺-dependent ADP ribosyltransferase (ART) mediating mitochondrial protein ribosylation; Sirt2

and 4 exert both NAD⁺-dependent HDAC and ART activities [34]. Although the enzymatic activity of Sirt7 as well as its specific substrates have not yet been determined, it has been shown that it resides in the nucleoli and regulates the RNA polymerase I (Pol I) transcriptional machinery [35].

The uniqueness of sirtuins is that their function as transcriptional regulators is directly linked to intracellular energetics. Accumulating evidence indicates that sirtuins participate in the coordination of several apparently disparate cellular functions such as cell cycle, response to DNA damage, metabolism, apoptosis, and autophagy [29]. These observations suggest that detailed characterization of the function of these enzymes under diverse cellular stress conditions will offer useful information towards designing novel compounds for therapeutic intervention in a wide range of apparently unrelated diseases including diabetes, neurodegenerative disorders, respiratory and cardiovascular diseases, and cancer. This paper will focus on Sirt1 since its physiological role has been more extensively studied, and the evidence regarding the activity of other sirtuins family members is still scarce.

2. Molecular Mechanisms Regulating Sirtuins Activity

Several studies have indicated the ability of the histone deacetylase III family members to deacetylate a wide range of substrates, thus implicating these enzymes in a broad spectrum of biological functions. Hence, considering the molecular circuits regulating sirtuins cellular levels (Figure 2) could provide the basic knowledge towards developing the means to control their accumulation for therapeutic benefit.

3. Enzyme Abundance

Sirtuins gene expression has been shown to be under the control of numerous transcription factors involved in the cell cycle regulation and apoptosis. Among them the oxidative stress and DNA damage responsive transcription factor E2F1, which induces cell cycle progression from G1 to S phase, directly binds to the Sirt1 promoter upregulating its gene expression in cells treated with the topoisomerase II inhibitor etoposide [36]. E2F1 phosphorylation by the stress-responsive kinase ataxia telangiectasia mutated (ATM) appears to be a prerequisite for E2F1-mediated regulation of Sirt1 gene expression [36]. In turn, the deacetylation function of Sirt1 inhibits E2F1's transcriptional activity [36].

The tumor suppressor p53, which is one of the most extensively mutated proteins in cancers, is a stress-responsive transcription factor that has also been shown to affect Sirt1 gene expression. Two functional p53-binding sites have been identified in the regulatory region of the Sirt1 promoter, and a complex regulatory network has been described to elucidate the modulation of Sirt1 gene expression in mammalian starved cells [37]. In particular, the activated in nutrient-deprived mammalian cells, forkhead box O transcription factor FOXO3a forms a complex with p53, which is recruited to the two p53-binding sites present within

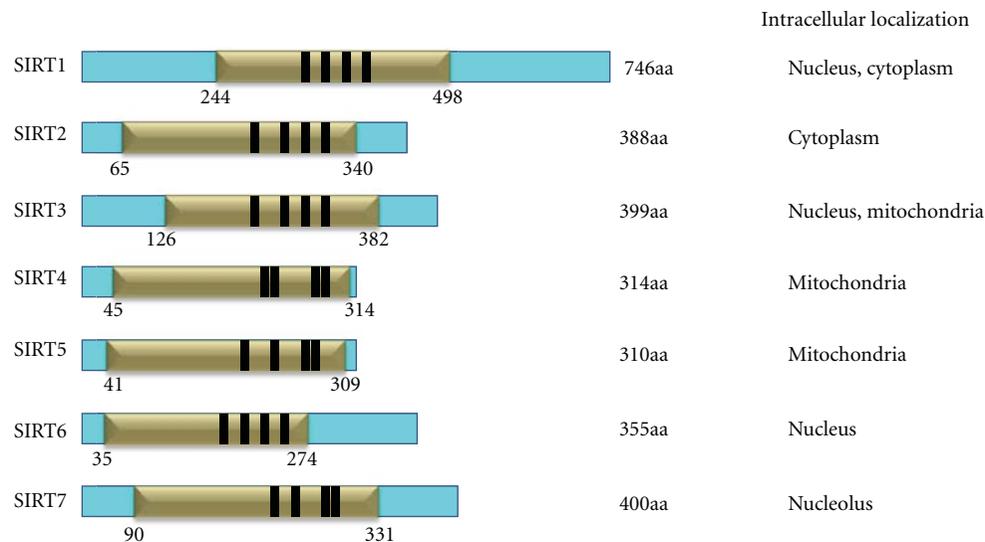


FIGURE 1: Schematic representation of human sirtuins family members 1–7, NAD-dependent catalytic domain (gold) (NAD-binding pocket), zinc-binding domain (black), and their intracellular localization.

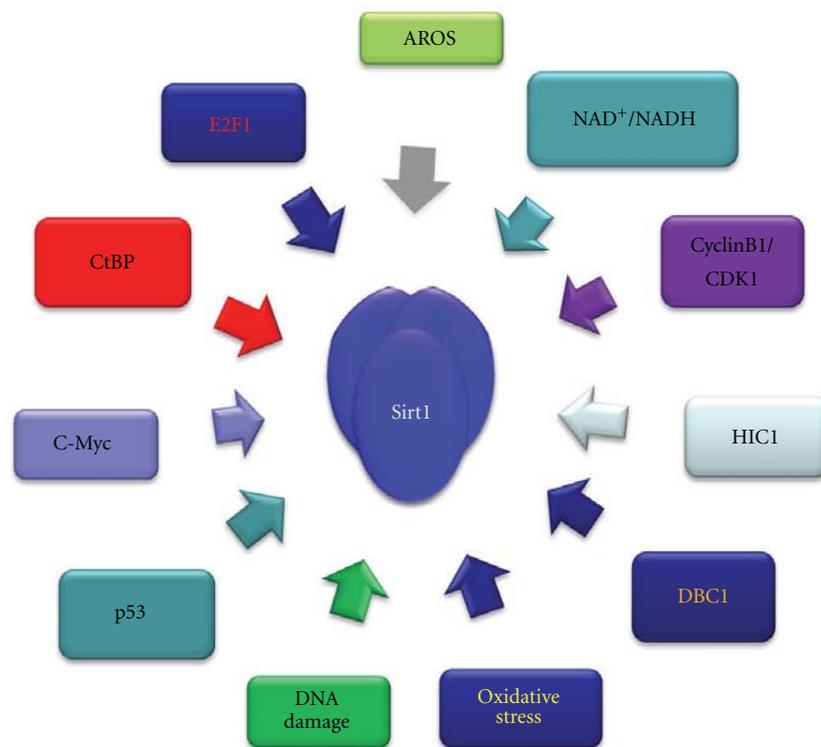


FIGURE 2: Factors involved in the regulation of Sirt1 gene expression and enzymatic activity.

the Sirt1 promoter, thus stimulating Sirt1 gene expression [37]. On the contrary, in normal nutrient conditions, p53 mediates repression of Sirt1 gene expression [37], which is a result of functional cooperation between p53 and the epigenetically regulated repressor hypermethylated in cancer 1 (HIC1) [38]. Thus, transcriptional activity and tumor suppressor functions exerted by p53 are indirectly regulated by HIC1 mediated repression of Sirt1 gene expression [39].

Furthermore, c-Myc upregulates Sirt1 gene expression, and in turn Sirt1-mediated c-Myc deacetylation leads to c-Myc protein degradation [40–42].

Another recently reported pathway regulating Sirt1 gene expression in response to acute metabolic changes involves the fine tuning of the association between the redox sensor carboxy terminal of E1A-binding protein (CtBP) and HIC1 [43]. The transcriptional repression activity of CtBP depends

on NADH levels, and in particular high NADH levels promote CtBP dimerization as well as its interaction with other transcriptional repressors such as HIC1 [44–47]. Cellular redox changes sensed by CtBP alter the affinity of the CtBP for HIC1 leading to a reduction of CtBP recruitment to Sirt1 promoter and hence derepression of its gene expression [43].

Sirt1 cellular levels are regulated by both p53 and E2F1 not only at the transcriptional but at the translational level as well. MicroRNA 34a (miR-34a) and miR449a, which are the p53 and E2F1 transcriptional targets, respectively, have been shown to inhibit Sirt1 expression [48, 49] resulting in p53 acetylation and induction of p53-dependent apoptosis. In addition miR199a knockdown during normoxia has been shown to stabilize HIF-1 α and Sirt1, whereas miR199a overexpression downregulates prolyl hydroxylase 2 (PHD2) implying that miR199a regulates HIF-1 α levels by moderating Sirt1 and hence PHD2 activities [50]. Sirt1 cellular levels are also regulated by the RNA-binding protein HuR, which associates with Sirt1 mRNA leading to increased Sirt1 mRNA stability and, thus elevated Sirt1 protein levels [51].

Regarding other members of the sirtuins family, an estrogen-related receptor (ERR α) responsive element (ERRE) has been mapped within the mouse Sirt3 promoter region, and colocalization of ERR α and peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α) has been confirmed in the Sirt3 promoter with chromatin immunoprecipitation assay [52].

4. Catalytic Activity

Sirtuins enzymatic activity is regulated by posttranslational modifications. *In vitro* evidence indicates that dephosphorylation at specific sites targeted by cyclinB/Cdk1 in a cell-cycle-dependent manner reduces its deacetylase activity [53, 54]. Sirt1 is also phosphorylated by the c-Jun N-terminal kinase 2 (JNK2) [55] and casein kinase 2 (CK2) [56]. JNK2-mediated phosphorylation of Sirt1 is associated with the regulation of its protein stability [55]. Both Sirt1 and CK2 are key regulators of similar biological functions including chromatin remodeling, cell cycle progression, and survival or apoptosis [53]. Multiple conserved phosphorylation sites have been identified within Sirt1 that are potential targets for a variety of kinases such as ATM, casein kinase 1 (CK1), DNA-dependent protein kinase (DNA-PK), extracellular-signal-regulated kinase (ERK1), glycogen synthase kinase 3 (GSK3), I κ B kinase (IKK), and mitogen-activated protein kinase (MAPK) [54]. Whether these kinases phosphorylate only Sirt1, other members of the sirtuin class, or Sirt1 phosphorylation by one of these kinases affects other posttranslational modification events, and Sirt1 substrate selectivity is not known. Recently two members of the dual-specificity tyrosine phosphorylation-regulated kinases (DYRK) DYRK1A and DYRK3, which play important role in body growth and brain physiology, have been demonstrated to promote cell survival by phosphorylating Sirt1 and inducing its deacetylase activity [57].

Like Sirt1, Sirt2 is phosphorylated by Cdk1 indicating that this sirtuin family member is also involved in the control of cell cycle. Additional evidence for that is provided by the fact that cyclinE/Cdk2 complex phosphorylates Sirt2 inhibiting its catalytic activity. Since cyclinE/Cdk2 is involved in the regulation of the cell cycle progression from G2 to M phase, inhibition of Sirt2 deacetylase activity mediated by cyclinE/Cdk2 might be a requirement for the cell cycle progression from G2 to M phase [58]. On the other side, overexpression of the CDC14A and CDC14B phosphatases, which are required for efficient DNA repair, inhibits Sirt2 protein degradation and interferes with adhesion and cell migration [59].

The active regulator of Sirt1 (AROS) is a 142 amino acid protein localized in the nucleus that interacts with Sirt1 and activates its deacetylase function [60]. Although the molecular mechanism of AROS-mediated activation of Sirt1 has not been defined, AROS possibly displaces a Sirt1 inhibitor from the deacetylase complex such as the deleted in breast cancer 1 (DBC1) inhibitor, or it recruits another cellular factor which induces a conformational change that activates Sirt1 enzymatic activity [61–63]. It has been shown that the binding affinity between Sirt1 and DBC1 is critical for the determination of cancer cell survival or death [64].

As is the case for other classes of deacetylases, acetylation is another posttranslational modification affecting the activity of various sirtuin members. For example, p300 acetylates Sirt2 and attenuates its deacetylase activity [65]. The molecular mechanism of p300-mediated inactivation of Sirt2 is not clear, but acetylated Sirt2 might acquire different conformation that alters its interaction pattern or affinity with other proteins which facilitate its association with proteins such as the 14-3-3 β/γ . Interaction of Sirt2 with 14-3-3 β/γ might influence its subcellular localization, thereby changing its activity [65].

Sumoylation of Sirt1 has been demonstrated to activate its deacetylase activity and occurs in the absence of DNA damage [66]. Exposure of cells to diverse types of stress conditions such as UV irradiation or hydrogen peroxide results in Sirt1 desumoylation mediated by the desumoylase sentrin-specific protease 1 (SEN1) and inactivation of its deacetylation function. As a consequence the proapoptotic Sirt1 substrates such as p53 are acetylated and hence active and capable to induce cell death [61, 67].

5. Availability of Metabolic Cofactors

The availability of NAD⁺ in cells is a limiting step in the activation of sirtuins catalytic activity since these enzymes require NAD⁺ as a cofactor to exert their function [68]. The basal intracellular NAD levels are maintained relatively constant [69] by the NAD biosynthetic and salvage pathways [70]. The precursor of the biosynthetic pathway of NAD synthesis is tryptophan and nicotinic acid (NA) or nicotinamide (NAM) the precursors of the salvage pathway [71, 72]. Human cells produce NAD⁺ by converting NAM in a two-step reaction catalysed by nicotinamide phosphoribosyltransferase (Nampt) [73]. The first step involves

the conversion of NAM to nicotinamide mononucleotide (NMN) by Nampt. NMN is subsequently utilized by nicotinamide/nicotinic acid mononucleotide adenyltransferase (Nmnat 1, -2, and -3) to regenerate NAD⁺ [74]. The molecular mechanism of nicotinamide-mediated inhibition of the sirtuins deacetylase activity has been elucidated in recent reports [75, 76]. Deficiency of the NAD⁺ synthesizing pathways abolishes sirtuins-mediated deacetylation [74] whereas increased NAD⁺ levels induce their enzymatic function [77]; therefore, by consuming NAD⁺ in order to exert their effects, sirtuins regulate the fluctuation of the NAD⁺/NADH ratio, thereby sensing cellular NAD⁺ concentration and redox status. For more detailed review of the relation between sirtuins NAD⁺/NADH ratio and oxidative stress see references [77–79].

To summarize, glucose deprivation and metabolic changes associated with calorie restriction alter the NAD⁺/NADH ratio [80–83]. Since sirtuins associate with chromatin and their function is NAD⁺-dependent, these enzymes couple changes of the metabolic flux and NAD⁺ levels with transcription [81].

6. Transcription Factors Associated with Sirtuins

Crucial cellular pathways involved in cell growth, differentiation, stress resistance, migration, and metabolism are modulated by the function of transcription factors whose activity is regulated by sirtuins including p53 [84–86], FOXO proteins [87], peroxisome proliferation-activating receptor-(PPAR-) gamma coactivator-1 α (PGC-1 α) [88], and nuclear factor- κ B (NF- κ B) (Figure 3 and Table 1) [34, 89, 90].

Several lines of evidence converge to the conclusion that sirtuins are integrated in the p53 pathway, and their function depends on the cellular status of p53 [84–86]. Sirtuins and p53 interact at various levels to induce cell cycle progression, senescence, or apoptosis [85] (Figure 3). The interplay between HIC1 and p53 regulates Sirt1 gene expression, and reduction or ablation of HIC1 can lead to tumorigenesis through Sirt1-mediated deacetylation and transcriptional inactivation of p53 [84, 85]. Sirt7 has also been shown to interact with p53 in the mice myocardium [91]. Apart from the acetylation levels, sirtuins regulate the subcellular localization of p53, thus determining the cellular fate under oxidative stress conditions [92].

Acetylation of HIF-1 α by arrest-defective protein 1 (ARD1) [93] and p300/CBP-associated factor (PCAF) [94] plays important role in the regulation of the protein stability and transcription target selectivity [94] of this transcription factor. Sirt1 deacetylates and represses HIF-1 α transcriptional activity [95] whereas Sirt1-mediated deacetylation of HIF-2 α induces its signaling in hypoxic conditions [96] suggesting that sirtuins signaling promotes the distinctive function of HIF-1 α and HIF-2 α [94, 96, 97]. Given the fact that HIF-1 α and HIF-2 α play crucial roles in the cellular adaptation to metabolic stress by regulating the expression of several genes involved in glucose metabolism, it is possible that the extent of their acetylation determines the pathway

of cellular energy production and redox balance depending on the type of tissue and environmental stress [90, 94–96, 98]. In accord with this perception, Sirt6 is recruited by HIF-1 α to histone 3 and deacetylates H3 lysine 9 within the promoter regions of several glycolytic genes repressing their gene expression, thereby regulating glucose homeostasis [98, 99].

The mammalian redox responsive FOXO transcription factors provide another example of the role of sirtuins in the determination of the cellular fate under oxidative stress conditions [104, 111]. Sirtuins target FOXO transcription factors under conditions of oxidative stress and determine their subcellular localisation, protein stability, and transcriptional activity [104, 111]. FOXO are involved in the cell cycle arrest at the G1-S and G2-M checkpoints [112], in scavenging reactive oxygen species (ROS) [113], and in the induction of the expression of genes involved in the DNA damage response, differentiation, glucose metabolism, and apoptosis [112, 114]. Sirt1-mediated deacetylation of FOXO3 and FOXO4 under stress induces cell cycle arrest instead of apoptosis [105]. Sirt2 and Sirt3 have also been shown to associate with FOXO transcription factors modulating their transcriptional activity and subcellular localization [107, 115–119]. Therefore, sirtuins by controlling the function of FOXO transcription factors indirectly exert a pivotal role in the regulation of multiple cellular processes [104] (Figure 3 and Table 1).

The E2F family of transcription factors is involved in the control of cell cycle progression, DNA damage response, and induction of apoptosis [120]. The transition from G1 to S phase checkpoint involves tight regulation of the E2F transcriptional activity by the pocket proteins retinoblastoma (pRb) tumor suppressor, p107 and p130. Acetylation of E2F1 by PCAF facilitates the binding of this transcription factor to its conserved DNA responsive elements and activation of gene expression of its targets, including several proapoptotic factors such as Apaf-1, Bim, caspase 7, and p73 [121]. Sirt1 binds and deacetylates pRb engaging the pRb tumor suppressor pathway in the oxidative stress-response [122–124].

Various subunits of the NF- κ B family of transcription factors are acetylated at multiple sites, affecting the DNA-binding and transcriptional activity of these proteins, thus modulating the release of proinflammatory mediators [125]. The p65/RelA subunit physically interacts and is deacetylated by Sirt1 resulting in the inhibition of the NF- κ B-mediated transcription [89, 90, 126, 127]. Deacetylation of NF- κ B and inhibition of its transcriptional activity by Sirt1 and Sirt6 protects pancreatic β cells from NF- κ B inflammatory response and preserves insulin secretion [99, 128].

7. Cellular Response to Stress

Sirtuins substrates are involved in the coordination of cellular responses to diverse stresses including inflammation, hypoxic stress, and heat shock, thereby regulating cell survival or death, differentiation, and endocrine signaling. In particular, sirtuins regulate the transcriptional activity of

TABLE 1: Transcription factors associated with sirtuins.

Sirtuin class	Substrate	Position	Function	Reference
SIRT1	p53	K120	Induction of cell cycle arrest	[100]
		K372	Unknown	[86]
		K382	Reduction of apoptosis	[86, 101]
	HIF-1 α	K674	Negative effect on tumor growth and angiogenesis	[95]
	FOXO1	K242, K245 and K262	Transcriptional activation	[102]
		Not known	Inhibition of FOXO1 activity	[103]
	FOXO3a	Not known	Induction of cell cycle arrest and resistance to oxidative stress; inhibition of FOXO-mediated induction of apoptosis; inhibition of FOXO transcriptional activity	[104, 105]
E2F1	Not known	Inhibition of E2F1 transcriptional activity; inhibition of E2F1-mediated apoptosis	[36]	
NF- κ B	K310 of RelA/p65 subunit	Inhibition of NF- κ B transcriptional activity and prevention of the release of proinflammatory mediators	[89]	
Sir2 α	p53	Not known	Attenuation of p53-mediated transcriptional activity Inhibition of p53-dependent apoptosis in response to DNA damage	[106]
SIRT2	FOXO3a	Not known	DNA binding and activation of target genes	[43, 107]
SIRT3	AceCS2	K642	Activation of the acetyl-CoA synthetase activity of AceCS2	[108, 109]
SIRT5	PGC-1 α	Not known	Unknown	[110]
SIRT6	HIF-1 α	Not known	Regulation of glucose homeostasis. Reduction of glycolysis and increase of mitochondrial respiration	[98]
SIRT6	NF- κ B	Not known	Reduction of NF- κ B-mediated apoptosis and senescence	[99]

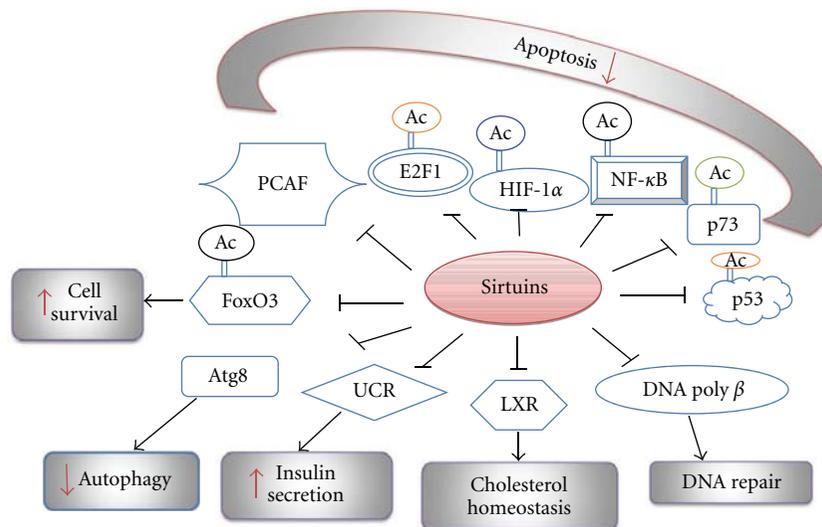


FIGURE 3: Sirtuins regulate the activity of numerous transcriptional regulators indirectly affecting the outcome of several cellular functions.

NF- κ B, p53, HIF-1 α , HIF-2 α , FOXOs, E2F1, and heat shock factor protein1 (HSF1), which are involved in the regulation of aging and aging-related diseases.

8. DNA Damage

In order to protect themselves from the high incidence of damage that could lead to mutations, genomic instability, or cell death and the different types of DNA damage that might occur, eukaryotic cells have developed appropriate mechanisms to detect and repair their damaged DNA. Efficient repair of the damage requires that the DNA repair machinery circumvents the barrier formed by the histone and nonhistone proteins that package DNA into chromatin and accesses the damaged site in a timely manner. In this respect, chromatin-remodeling and histone-modifying enzymes are crucial for the ability of eukaryotic cells to detect and repair DNA breaks. Consistent with this notion, several reports have indicated that acetylases and deacetylases are recruited in the vicinity of DNA breaks [129].

Direct indication for the involvement of sirtuins in DNA damage response has been revealed in Sirt1^{-/-} and Sirt6^{-/-} mice which exhibit increased radiation sensitivity, chromosomal aberrations, and impaired DNA repair [130, 131]. The involvement of sirtuins in the DNA damage response was initially suggested by observations demonstrating increased chromatin recruitment of Sirt1 to sites of DNA-DSB in mammalian cells upon diverse types of DNA damage including oxidative stress in a manner involving stress-responsive kinases such as ATM [132–134]. Upon DNA damage, Sirt1 deacetylates the Nijmegen breakage syndrome (NBS1) which is a DNA-DSB sensor and repair protein, thus facilitating the recruitment of other required factors to the sites of damaged DNA and optimal repair through homologous recombination (HR) and nonhomologous end joining (NHEJ) [135]. Sirt1 is also required for the optimal function of the nucleotide excision repair (NER) pathway. In particular, it has been shown that Sirt1 impairs NER by suppressing the xeroderma pigmentosum C (XPC) gene expression which is essential for the recognition of DNA lesions and NER initiation [136]. Overexpression of Sirt1 represses proteins with DNA damage repair functions such as various FOXO family members [105], Ku70 [137], p73 [138], pRb [122], and Werner helicase (WRN) [139]. Further evidence for the involvement of Sirt1 in the DNA damage response is provided by the fact that it is transcriptionally upregulated by breast cancer 1 early onset (BRCA1), which binds to DNA-DSB and plays a significant role in DNA repair and the maintenance of genomic stability [140]. Overall, Sirt1 is involved in DNA damage response by modulating the expression of genes involved in DNA repair and by recruiting to sites of DSBs factors participating in the processing of DNA damage.

Sirt6^{-/-} mice display sensitivities associated with deficiencies in base excision repair (BER) such as genomic instability and enhanced sensitivity to ionizing radiation and DNA damaging agents [131]. The detailed molecular mechanisms by which Sirt6 regulates DNA damage repair

has been suggested to involve the function of the DNA-PKcs which is a kinase that takes part in the NHEJ [141]. Sirt6-mediated deacetylation of the H3K9 at sites surrounding DSBs allows DNA-PKcs or other repair factors to access the DNA lesions [131]. Sirt6-dependent deacetylation of the C-terminal-binding protein (CtBP)-interacting protein (CtIP) which promotes DNA end resection and is required for efficient homologous recombination is another proposed mechanism for SIRT6-dependent processing of DNA damage repair [134, 142]. Consistently with the role of other chromatin-modifying enzymes, Sirt6 in response to DNA damage is recruited to DNA breaks either genome-wide or locally contributing directly to DNA damage repair or indirectly by permitting access to the DNA lesions to the DNA damage repair machinery.

Further research is required to characterise the molecular networks linking transcription and chromatin modifications to DNA damage response and repair as well as to elucidate the role of other sirtuin family members in these processes. It will also be interesting to determine whether different sirtuin family members are involved in the same or diverse DNA damage and repair pathways and whether they function in concert or exert antagonistic effects.

9. The NAD⁺/NADH Ratio

During glycolysis and citric acid cycle, energy from nutrients is transferred to NAD⁺ which is reduced to NADH. NADH is then oxidized back to NAD⁺ by transferring its reducing electrons to electron acceptors and ultimately to oxygen, and the energy released during this process is coupled to ATP generation through oxidative phosphorylation (OXPHOS) [77]. Increasing evidence suggests that marked alterations in the NAD⁺/NADH ratio may have detrimental effects on the cellular fate; therefore, NADH levels are very tightly regulated in cells [143]. High cytosolic NADH levels result in reactive oxygen species (ROS) generation and oxidative damage by several mechanisms including providing substrates for NAD(P)H oxidase and release of iron from ferritin. Furthermore, high levels of cytosolic NADH can either promote OXPHOS by increasing the mitochondrial NADH levels or inhibit OXPHOS by promoting pyruvate to lactate conversion and reducing the permeability of the voltage-dependent anion channel (VDAC) in the mitochondrial outer membrane [143]. Since the effects of altered cytosolic NADH levels on cell injury are complicated, further studies are required to resolve this issue.

Sirtuins consume NAD⁺ in order to exert their enzymatic functions, and by doing so they alter the NAD⁺/NADH ratio, thereby modifying the redox status within the cells and hence serving as cellular sensors of reduced glucose and NAD⁺ availability [23, 67, 74, 75]. Changes in energy or increase of the NAD⁺/NADH ratio enhances sirtuins activity and protein deacetylation implying that there is a close association between the cellular redox status and the acetylation levels within the cells [68]. Indeed, Sirt1 has been shown to determine survival or apoptosis of renal tubular cells by fine tuning the ROS scavenger catalase gene expression

in the presence or absence of intracellular ROS [144]. In particular, in the presence of high levels of intracellular ROS, Sirt1 induces FOXO3a-mediated upregulation of catalase gene expression leading to reduction of oxygen consumption and ROS levels leading to cell survival [144–146]. Reduction of ROS levels by Sirt1 is the mechanism underlying the development of resistance to oxidative stress which is associated with aging and diseases such as type 2 diabetes mellitus [145]. Sirt2-mediated deacetylation of FOXO [107, 116] and Sirt3-dependent decrease of ROS production in brown adipocytes [147] as well as Sirt3 involvement in antioxidants production and NADPH regeneration [148] suggest that several sirtuin family members play important role during oxidative stress. More work is needed to characterise the detailed mechanisms by which sirtuins contribute to the control of cellular redox levels, which potentially will lead to the development of novel therapeutic approaches for the treatment of diseases typified by high-inflammatory states.

10. Cell Cycle

The involvement of sirtuins in the sensitisation and repair of DNA damage as well as the regulation of the cellular redox state implies that these enzymes are involved in the control of the cell cycle in order to provide the necessary time for the cells to repair their DNA damage under conditions of oxidative stress. Multiple transcription factors playing critical role in the control of cellular proliferation and apoptosis have been identified as sirtuin substrates (Table 1); therefore, changes in sirtuins cellular levels affect the ability of cells to divide. Sirtuins exert both positive and negative effects on cell growth promoting and inhibiting cellular proliferation. Many studies converge to the conclusion that inhibition of sirtuins is beneficial in cancer treatment, which is consistent with the negative effects they mediate mainly on the tumor suppressor p53 preventing senescence and programmed cell death [149]. Furthermore, increased expression of sirtuins in cancer cells coincides with pRb hyperphosphorylation and p16^{INK4A} downregulation [150, 151].

Cell cycle regulation through Sirt1 is carried out by the deacetylation of three members of the FOXO family of transcription factors, namely FOXO1, FOXO3a, and FOXO4 [104, 152]. Deacetylation of FOXO alters their interaction pattern with E2F1 or p53, leading to cell cycle arrest or apoptosis in a manner dependent on environmental conditions, tissue characteristics, and cellular metabolic state [152]. In response to oxidative stress, Sirt1 deacetylates FOXO3a increasing its ability to cause cell cycle arrest and preventing it from inducing apoptosis [104]. Cell cycle arrest is also the result of Sirt1-mediated deacetylation of FOXO1 and FOXO4 which are activated by deacetylation and induce gene expression of the p27^{Kip1} cyclin/cdk inhibitor. In addition, deacetylation of these transcription factors induces gene expression of the antioxidant manganese superoxide dismutase (MnSOD) and the DNA repair gene growth arrest and DNA damage inducible (GADD45), thereby enhancing the cellular defence to oxidative stress [104, 152]. Taken together, these observations indicate that in response to

oxidative stress, Sirt1 selectively targets FOXO transcription factors to their prosurvival subset of transcriptional target genes, thereby inducing cell cycle arrest and resistance to oxidative damage. Apart from FOXO, Sirt1 inhibits cellular proliferation by destabilising c-Myc [40, 153].

The role of Sirt2 in cell cycle regulation has been elucidated in cells overexpressing Sirt2 where it acts as a G2/M checkpoint regulator preventing chromosomal instability [53, 59, 154]. Sirt2 has also been shown to deacetylate tubulin and cause cell cycle arrest prior to entry into mitosis in response to microtubule inhibitors such as nocodazole [155].

Studies in Sirt3 null mouse embryonic fibroblasts have indicated that the main function of this sirtuin family member is the regulation of the mitochondrial ROS levels [156]. Sirt3 is normally localised inside the mitochondria but in response to cellular stress, it can be detected in the nucleus where it relocates in the presence of overexpressed Sirt5 [157]. In addition, Sirt3 and Sirt4 have recently been shown to exert antiapoptotic effects in response to DNA damage when the levels of NAD⁺ are extremely low [158].

Sirt6 is a key component of the base excision repair, but whether it delays cell cycle progression to allow time for DNA repair to occur, it is not known [131]. Sirt6 has been shown to associate with telomere maintenance and its depletion results in abnormal telomere structures, end-to-end chromosomal fusions, and premature senescence [159].

Sirt7 is localised in the nucleoli and is involved in the regulation of the RNA polymerase I and the transcription of the ribosomal gene (rDNA). During mitosis, Sirt7 is phosphorylated and retains its nucleolar localisation until telophase when it is dephosphorylated and activated to resume rDNA transcription [160].

In summary, the detailed role of all sirtuin family members in the regulation of the cell cycle requires further investigation in order to understand the link between cell cycle control and their ability to regulate transcription and acquire diverse subcellular localisation during different cell cycle phases. Moreover, it is intriguing to investigate whether each member of the sirtuins family exerts its effect on cell cycle individually or in combination with other sirtuin family members or other cell cycle regulators.

11. Apoptosis

Sirt1 interacts, deacetylates, and thereby negatively regulates the transactivation function of various key transcription factors playing central role in the determination of the cellular fate (apoptosis/survival) such as p53 [85], E2F1 [36], members of the FOXO family of transcription factors [111], NF- κ B [89], HIF-1 α [95] and the DNA-PKcs subunit, and DNA damage end-joining protein Ku70 [161] (Table 1). Sirt1 deacetylates several sites of the tumor suppressor p53 including K320, K372, and K382, which are selectively acetylated by PCAF and p300 under diverse stress conditions, thus antagonising senescence or inducing either cell cycle arrest or apoptosis [94]. Hyperacetylation of p53 enhances its transactivation function consequently leading to the increase of the gene expression of its proapoptotic targets.

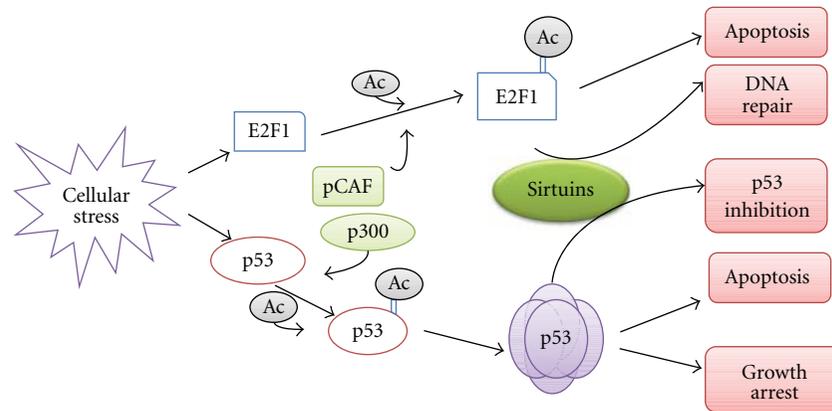


FIGURE 4: Role of sirtuins in the cellular response to stress.

Sirt1-dependent deacetylation of p53 reduces the ability of p53 to induce gene expression of its proapoptotic targets, thereby suppressing apoptosis in response to oxidative stress and DNA damage ([85] and Figure 4). Besides the control of p53 activity, Sirt1 regulates the proapoptotic function of the proapoptotic p53 target Bax by retaining it in the cytoplasm in complex with the hypoacetylated form of Ku70 and preventing it from translocating to the outer mitochondrial membrane to induce apoptosis [162, 163]. Upon cellular stress conditions such as UV irradiation, Ku70 is acetylated by PCAF and dissociates from Bax which is now able to induce cell death [163].

Similarly to p53, the activity of FOXO transcription factors is regulated by acetylation and deacetylation modifications. Sirt1 inhibits the ability of FOXO3a to promote apoptosis by blocking gene expression of the proapoptotic Bim, Puma, and TRAIL which are FOXO3a transcription targets in a cellular context manner [161, 164]. Furthermore, Sirt1 regulates cellular apoptotic responses through the E2F1/p73 pathway [121], and it is important in controlling cell death in hypoxic and heat shock conditions by increasing the activity of HIF-2 α [96] and heat shock factor 1 (HSF1) [165]. Cleavage of Sirt1 by caspase is another possible pathway through which this deacetylase induces apoptosis, but the necessity of this cleavage for the initiation of apoptosis has not been confirmed [166, 167].

It appears that Sirt1 has both negative and positive effects on apoptosis since by deacetylating p53 and FOXO transcription factors, it suppresses apoptosis [168] whereas in response to TNF α signaling by deacetylating RelA/p65, it inhibits the transcriptional activity of the antiapoptotic transcription factor NF- κ B [61, 89].

The role of Sirt3 in apoptosis appears to be more complicated since this sirtuin family member has been found to cause cell death in several nonstressed human cancer cell lines in which Bcl-2 had been silenced [169] as well as in myeloid leukemia cell lines treated with the natural flavonoid kaempferol [170], whereas both Sirt3 and Sirt4 have been shown to be required for the maintenance of the mitochondrial NAD⁺ in genotoxic stress conditions and hence cell survival [109, 158].

It has recently been demonstrated that Sirt5 interacts with cytochrome c in the mitochondrial membrane [148, 171] but the functional consequences of the Sirt5-dependent deacetylation of cytochrome c and in particular whether this modification has any impact on apoptosis have not yet been elucidated.

Sirt7 depletion by RNAi led to inhibition of cell growth and induction of apoptosis in U2OS cells [35] and primary cardiomyocytes [91] postulating that this sirtuin family member is also essential for cell survival [35, 160] although its effects are probably tissue-type specific.

Taken together the aforementioned observations imply that the design of sirtuin inhibitors to promote apoptosis should take into consideration whether the targets deacetylated by sirtuins are deacetylated by members of other HDAC families, that sirtuins might have both positive and negative effects on apoptosis, and that sirtuins do not enhance to the same extent the transcriptional activity of all the genes induced by the same transcription factors, and it might not be sufficient to block their catalytic function to achieve induction of cell death.

12. Autophagy

Autophagy is a cytoprotective process by which eukaryotic cells degrade damaged or dysfunctional organelles and proteins with long half-life. The degradation takes place in the lysosome where the cytoplasmic contents to be degraded are delivered enclosed in double-walled membrane vesicles called autophagosomes which originate from the endoplasmic reticulum [172–174]. The fact that the efficiency of the autophagic degradation declines during aging and the efficient maintenance of autophagy leads to lifespan extension attracted the interest of several research laboratories to investigate the signaling pathways regulating autophagy with the aim to understand the factors involved in the lifetime determination and the causes of age-related degenerative diseases. Recent research efforts have shed light on the biochemical autophagic machinery and the crucial role of several genes called autophagy-related (Atg) genes in the execution of this process [175–177].

Sirtuins are important longevity factors as well; therefore, it was hypothesised that lifespan might be determined by interacting signaling network pathways regulating sirtuins functions and autophagy. Support to this hypothesis was lent by the observation that *Sirt1*^{-/-} and *Atg5*^{-/-} deficient mice exhibit partially similar phenotypes as well as that *Sirt1*^{-/-} mouse embryonic fibroblasts under starved conditions are unable to activate autophagy [178]. In addition, transiently expressed wild type *Sirt1* but not its inactive deacetylase mutant could stimulate basal levels of autophagy [178]. The molecular mechanisms entailed in *Sirt1*-mediated regulation of autophagy have not yet been elucidated, but it has been proposed that *Sirt1* induces autophagosome formation by associating and deacetylating the *Atg5*, *Atg7*, and *Atg8* components of the autophagic machinery in a NAD^+ -dependent manner, thus facilitating the assembly of *Atg* complexes [172, 178–183].

Apart from its direct effects on the components of the autophagic apparatus, *Sirt1* associates with well-characterized mediators of autophagy and lifespan such as mTOR (target of rapamycin) [184] FOXO transcription factors [111, 183] p53 [185] and E2F1 [186]. The molecular mechanisms by which autophagy is negatively regulated by mTOR are not well established, nevertheless it has been reported that mTOR inhibition or *Sirt1* activation prolong lifespan [173, 187]. *Sirt1* and FOXO3 interact in response to oxidative stress, and *Sirt1* deacetylates and activates FOXO3 which in turn induces the expression of many autophagy-related genes stimulating autophagy and cellular stress resistance [111]. Several signaling pathways link autophagy and the tumor suppressor p53, which has been shown to exert both negative and positive effects on this process [188, 189]. It appears that only cytoplasmic p53 can induce autophagy in a manner involving the E3 ligase activity of HDM2 [189]. It is not known whether *Sirt1* exerts its effects on autophagy through p53 by affecting the tumor suppressor's subcellular localisation or protein stability [190]. E2F1 is a transcriptional regulator of autophagy as it upregulates the expression of several ATGs as well as the damage-regulated autophagy modulator (DRAM) [186]. Since *Sirt1* modulates E2F1's transcriptional activity [36, 121], it is possible that *Sirt1*-determined acetylation status of E2F1 mediates its effects on autophagy.

There are not many reports in the literature that address the role of the other sirtuin family members in autophagy apart from *Sirt2* that has been shown to deacetylate FOXO1 and dissociate its complex with *Atg7* leading to apoptosis [191]. Other open questions in relation to the regulation of autophagy by sirtuins are whether increasing autophagy by activating sirtuins would have beneficial effects in terms of longevity, or it would lead to side effects by affecting other vital cellular functions in which sirtuins have been implicated.

13. Sirtuins in Pathology

Sirtuins are key components of a broad range of biological processes, which are directly or indirectly linked to aging.

Sirtuins can regulate the aging process partly through their ability to connect the nutritional status of the cell to chromatin modifications and regulation of gene expression [192, 193]. Their role as longevity mediators is due to their activity as modulators of several calorie restriction (CR) pathways [192]. CR extends life span by shifting the glucose metabolism toward respiration [68] followed by possible alterations of the NAD^+/NADH ratio, modulation of the sirtuins deacetylase or ADP ribosyl-transferase activity [81], and slow rate of ROS generation which correlates with increased longevity [194]. Aging process and several age-related diseases such as diabetes [128, 195], cardiovascular [196], neurodegenerative [197], respiratory [198], and autoimmune diseases [12], and cancer [22, 29, 34, 69, 199] on the other side are accompanied by elevated redox cellular content or low-grade chronic, proinflammatory stress [32]. In view of the fact that sirtuins are involved in the regulation of the aging process and in sensing oxidative stress, it has been suggested that sirtuins could play a vital role in the development of a variety of aging diseases and their function could be targeted for therapeutic benefit in these diseases. Here the role of the most extensively studied member of the sirtuin family, *Sirt1*, in tumorigenesis and as a target for the development of anticancer therapeutics will be discussed.

14. *Sirt1* in Oncogenesis

The role of *Sirt1* in tumorigenesis appears to be complex as this protein has been associated with oncogenic and tumor suppressor function, and both elevated and decreased levels have been detected in different types of cancer suggesting that *Sirt1* functions in tumorigenesis are tissue-type and context specific [149, 200, 201]. For example, increased *Sirt1* levels have been identified in acute myeloid leukaemia (AML) [202] and colon cancer where *Sirt1* might promote proliferation and survival [203] or cell growth inhibition [204]. Both increased and reduced levels of *Sirt1* have been detected in prostate cancer [130, 205] and decreased levels in ovarian and glioma cancers [37, 130] which is possibly due to defects in the activity of tumor suppressor genes that regulate its gene expression [38].

Sirt1 determines changes in gene transcription and most of its effects in oncogenesis by deacetylating and thus regulating the function of a large number of tumor suppressors or oncogenes (Table 1) such as p53 [86, 106], FOXO transcription factors [104, 105], p73 [206], pRb [122], PML [101], Ku70 [163], hTERT [207] MyoD [208], NF- κ B [209], and BCL6 [210].

Sirt1 is a direct effector of p53 transcriptional activity regulating the gene expression of p53 targets involved in cell cycle arrest (*CDKN1A* encoding p21^{WAF-1/CIP-1}) and apoptosis (Bax) under DNA damage conditions as well as p53 protein stability [84, 94]. In turn *Sirt1* gene expression is under the control of the p53-HIC1 loop [85, 211]. The precise role of *Sirt1* in tumorigenesis is, therefore, dependent on the presence and activity of p53 [149]. *Sirt1*-mediated deacetylation of FOXO has been shown to promote ubiquitination and degradation of these transcription factors

[12] as well as to induce FOXO-dependent cell cycle arrest and evasion of apoptosis in response to DNA damage [104, 105]. Deacetylation of pRb in a Sirt1-dependent manner has been suggested to reverse cell cycle arrest after DNA damage repair has taken place [122]. Another mechanism by which Sirt1 promotes cell survival is by deacetylating E2F1, thereby repressing its transcriptional activity and preventing the induction of the E2F1/p73 apoptotic pathway in response to DNA damage [121]. One of the major functions of Sirt1 is the regulation of inflammation through deacetylation of the proinflammatory transcription factor NF- κ B [209] and consequent modulation of gene expression of various cytokines (TNF α , ICAM-1 (intercellular adhesion molecule-1), IL-6 (interleukin-6), and IL-8) [32, 89, 213]. In addition, tumorigenesis might be affected by Sirt1 through its considerable regulatory effects on metabolic processes and the regulation of oxidative stress [214, 215].

Therefore, specific Sirt1 inhibitors for each type of tissue needs to be considered in order to increase acetylation in the case of inactivation of oncogenes or activation of the expression of proapoptotic genes.

15. Conclusions and Perspectives

Deacetylation reaction catalysed by class III deacetylases requires the consumption of NAD⁺ and links chromatin epigenetic changes and transcriptional regulation with energy metabolism. Another important difference between sirtuins and the other classes of deacetylases is that apart from deacetylase, some class III family members possess ADP-ribosyltransferase activity. The mitochondrial localisation of these enzymes and their involvement in acetylation/deacetylation processes of mitochondrial proteins is an additional indication of the coupling between metabolic networks and acetylation. These characteristics of the sirtuin family members implicate them in a wide range of diverse cellular processes ranging from glucose homeostasis, to cellular growth, senescence, stress resistance, and metabolism. This in combination with the fact that there are seven members of the sirtuins family raise the theoretical proposal that intervention in each one of the different cellular processes modulated by distinct members of the sirtuins family could increase the specificity of therapeutics by selectively targeting different sirtuins-mediated pathways. Consequently inhibitors specific for each member of the sirtuins family could provide a valuable tool towards understanding the link of individual sirtuin-member-specific biological functions to alternative pathological situations.

Although intensive research in recent years has provided many answers in relation to the role of sirtuins in human physiology, there are still many questions and controversies that require to be addressed. For instance, identification of the factors determining sirtuin members' tissue as well as organelle specific function and whether this is linked to aging or other physiological or pathological conditions could facilitate individualisation of treatment with sirtuins modulators. In addition, it is not known whether specific sirtuin family members can only function within particular

pathways, or overlapping synergistic or antagonistic effects between different members of the family could impact on the same pathways. Moreover, in the case of the sirtuin members exerting both deacetylase and ADP ribosyl transferase activity, the conditions determining the one or the other activity and the relative contribution of each one of these activities to the development of disease requires further investigation. Future research will also define the reason that Sirt1 overexpression in some cancer tissues has oncogenic effect and tumor suppressive in others.

Clearly there is a lot of work required to fully understand the complex role of sirtuins in human physiology and to hopefully identify new therapeutic uses of sirtuin activators and inhibitors.

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Review Article

Histone Deacetylases in Neural Stem Cells and Induced Pluripotent Stem Cells

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Stem cells have provided great hope for the treatment of a variety of human diseases. However, the molecular mechanisms underlying stem cell pluripotency, self-renewal, and differentiation remain to be unveiled. Epigenetic regulators, including histone deacetylases (HDACs), have been shown to coordinate with cell-intrinsic transcription factors and various signaling pathways to regulate stem cell pluripotency, self-renewal, and fate determination. This paper focuses on the role of HDACs in the proliferation and neuronal differentiation of neural stem cells and the application of HDAC inhibitors in reprogramming somatic cells to induced pluripotent stem cells (iPSCs). It promises to be an active area of future research.

1. Introduction

Stem cells have the ability to self-renew and differentiate into multiple lineages. Identifying regulators that control stem cell self-renewal and differentiation is essential for the development of stem cell-based cell replacement therapies for human diseases and injuries [1]. Epigenetic control, including histone modification, has been shown to play an important role in regulating both stem cell self-renewal and pluripotency [2–5].

Histone modification by acetylation is the most well-studied histone modification and has been shown to be an important means of gene regulation [6]. In general, acetylation of histone tail disrupts the electrostatic interaction between positively charged amino acids from the histone tail and negatively charged phosphate group in DNA, leading to decompression of chromatin structure. The open chromatin allows for the access of transcription factors and ultimately gene activation [7]. Acetylated histone tails may also serve as docking sites for the recruitment of bromodomain proteins, a class of transcriptional activator [8]. On the other hand, histone deacetylation leads to gene repression [9]. In addition, transcription factors themselves have also been shown to be regulated by acetylation and deacetylation,

respectively [10]. The histone acetylation is mediated by histone acetylases (HATs), while histone deacetylation is catalyzed by histone deacetylases (HDACs). HDACs have been shown to regulate many important biological processes, including cell proliferation, differentiation, and development, by forming complexes with various transcription factors and transcriptional coregulators [8].

Neural stem cells are stem cells of neural origin. They retain the ability to proliferate and self-renew and have the capacity to give rise to both neuronal and glial lineages [11–14]. A complete understanding of neural stem cells and neurogenesis requires the identification of molecules that determine the self-renewal and multipotent character of these cells. These molecules likely include epigenetic regulators, such as HDACs, that act to regulate stem cell self-renewal and differentiation by controlling the activity of a network of downstream target genes [15].

Recent breakthrough studies using retroviral transduction of a transcription factor quartet to reprogram human somatic cells into induced pluripotent stem cells (iPSCs) have led to an important revolution in stem cell research [16–18]. Comparative analysis of human iPSCs and human embryonic stem cells using assays for morphology, gene expression profiles, epigenetic status, and differentiation

potential have revealed a remarkable degree of similarity between these two pluripotent stem cell types. These advances in reprogramming will enable the creation of patient-specific stem cell lines to study various disease mechanisms. The cellular models created will provide valuable tools for drug discovery. Furthermore, this reprogramming system provides great potential to design customized patient-specific stem cell therapies with economic feasibility [19]. However, reprogramming by viral infection is a slow process with very low efficiency. Recent progress in using HDAC inhibitors to enhance reprogramming efficiency will be discussed.

2. HDACs in Neural Stem Cells

Neural stem cell self-renewal and differentiation are the result of transcriptional control in concert with chromatin remodeling and epigenetic modifications. During central nervous system development in vertebrates, neural stem cell fate is strictly controlled under regional and temporal manners, accompanied by precise epigenetic control [20].

We have shown that HDAC-mediated transcriptional repression is essential for the proliferation and self-renewal of neural stem cells (Figure 1) [21]. There are 11 HDACs in the HDAC superfamily [8]. Among them, HDAC1, HDAC3, HDAC5, and HDAC7 are highly expressed in neural stem cells [21, 22]. The expression of these HDACs is reduced upon differentiation. On the other hand, HDAC2 expression is more widespread in the brain [22]. While HDAC2 is expressed in proliferating neural progenitors, its expression is upregulated as neurons differentiate [22]. HDAC11 is also predominately expressed in mature neurons and minimally expressed in neural precursors [23].

We showed that HDACs function in neural stem cells through nuclear receptor TLX, an essential neural stem cell regulator [24]. Both HDAC3 and HDAC5 have been shown to be recruited to the promoters of TLX target genes in neural stem cells. Recruitment of HDACs led to transcriptional repression of TLX target genes, the cyclin-dependent kinase inhibitor p21, and the tumor suppressor gene pten. Disruption of the TLX-HDAC interaction led to substantial induction of p21 and pten gene expression and dramatic inhibition of neural stem cell proliferation [21]. The role of p21 as an HDAC target in cell proliferation was also demonstrated in embryonic stem cells recently [25]. It is worth noting that p21 has been identified as a target for HDACs in earlier studies as well [26]. While most reports point to p21 as a target for HDACs independent of p53 [27, 28], a direct role for p53 in the induction of p21 expression has also been reported [29].

In addition to self-renewal, HDACs also regulate neural stem cell differentiation. Treatment of adult neural stem cells with HDAC inhibitors induced neuronal differentiation and upregulated neuronal-specific genes, such as NeuroD, neurogenin 1 (Ngn1), and Math1 (Figure 1) [30–34]. *In vitro* treatment of adult neural subventricular zone precursor cells with HDAC inhibitors also led to increased production of neurons, with concomitant induction of NeuroD, cyclin D1, and B-lymphocyte translocation gene 3 [33]. In addition

to its effect on adult neurogenesis, treatment of embryonic neural stem cells with the HDAC inhibitor trichostatin A (TSA) also led to increased neuronal differentiation, with decreased astrocyte differentiation [30].

Genetic studies using knockout mouse models revealed that HDAC1 is a major deacetylase in embryonic stem cells. HDAC1-null embryonic stem cells exhibit proliferation defects with increased expression of the cyclin-dependent kinase inhibitors p21 and p27 [35]. Furthermore, HDAC1 and HDAC2 play redundant and essential role in the progression of neuronal precursors to neurons. Deletion of both HDAC1 and HDAC2 led to a failure of neuronal precursors to differentiate into mature neurons [36]. On the other hand, HDAC2 is required to silence neural progenitor-specific gene expression during neuronal differentiation in the neurogenic zones of adult brains, as revealed by studies using either conditional deletion of HDAC2 or in mice lacking the catalytic activity of HDAC2 [37].

A wide range of brain disorders have been shown to be associated with the imbalance between the activity of HATs and HDACs [38]. Therefore, HDAC inhibitors have the potential to intervene neurodegenerative diseases. Indeed, HDAC inhibitors have been shown to exhibit neuroprotective effect, induce neurotrophic factor expression, display anti-inflammatory properties, and improve neurological performance in animal models of neurological diseases [38]. Considering the important role of HDACs in controlling neural stem cell proliferation and neurogenesis, HDAC inhibitors may emerge as novel therapeutic tools for the treatment of a variety of neurodegenerative diseases. As a proof-of-principle, the HDAC inhibitor valproate has been used as an anticonvulsant drug and mood stabilizer to treat bipolar disorder, a manic-depressive illness [39].

3. HDAC Inhibitors in iPSCs

In addition to neural stem cells that have offered great potential for the treatment of neurological diseases, pluripotent stem cells have also provided great hope for cell replacement therapies for neurological disorders and other degenerative diseases because of their ability to self-renew and their potential to form all cell lineages in the body [40]. Recently, an important revolution in stem cell research has been undertaken. Using a cocktail of four factors, somatic cells can be reprogrammed into iPSCs [16–18, 41–44]. iPSCs are very similar to embryonic stem cells in that both have the capability to self-renew and differentiate into all cell types, but iPSCs are produced from epigenetic reprogramming of somatic cells through the exogenous expression of four pluripotency-related transcription factors [45, 46].

iPSCs were first generated by retroviral transduction of four factors, Oct4, Sox2, Klf4, and c-Myc [17, 43]. Since then, the technique has been optimized and conducted in different ways, by selecting the cell types to be reprogrammed, using different combinations of reprogramming genes and improving the method for reprogramming factor delivery [46]. One of the most common reprogramming gene delivery methods is retroviral and lentiviral transduction. However, the efficiency of reprogramming using

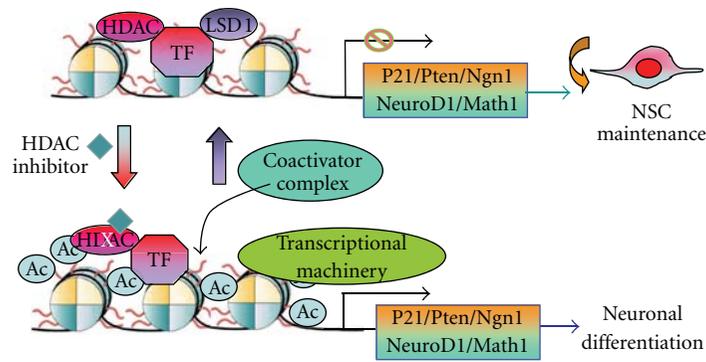


FIGURE 1: HDACs in neural stem cell proliferation and neuronal differentiation. In proliferating neural stem cells (NSCs), transcription factors (TF) recruit HDACs to the promoters of their downstream target genes, to repress the expression of cell cycle inhibitors, such as p21 and pten, and neuronal-specific genes, such as NeuroD, Neurogenin 1 (Ngn1), and Math 1, to maintain NSC proliferation and self-renewal. In addition to promote NSC proliferation, HDACs also inhibit neuronal differentiation. Treatment of HDAC inhibitors leads to induced neuronal differentiation, with increased expression of p21 and pten, and neuronal-specific genes. AC stands for histone acetylation.

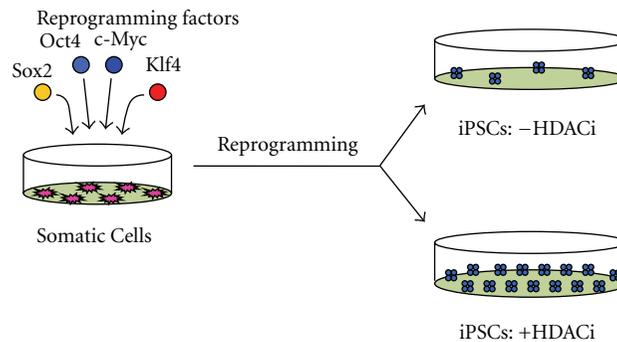


FIGURE 2: Treatment of HDAC inhibitors enhances reprogramming efficiency. When reprogramming somatic cells to iPSCs by ectopic expression of the four transcription factors (Oct4, Sox2, Klf4, and c-Myc), the resulting reprogramming efficiency is low (iPSC: -HDACi). But with the use of HDAC inhibitors (HDACi), the reprogramming efficiency can improve by more than 100-fold (iPSC: +HDACi), especially when using the HDACi, valproic acid (VPA). Through this improvement, it is suggested that histone modification plays an important role in inducing pluripotent stem cells.

viral vectors is extremely low and the overall process is slow [47]. Several lines of evidence support the notion that stochastic epigenetic events contribute to the low efficiency of reprogramming [45]. The observation that reprogramming is a slow and gradual process that takes several weeks further supports this notion.

Small molecules involved in epigenetic regulation, such as DNA methyltransferase inhibitors [48, 49], histone deacetylase inhibitors [47, 48], and histone methyltransferase inhibitors [50], have been shown to improve the efficiency of reprogramming substantially. The histone deacetylases have been shown to enhance the efficiency of reprogramming mediated by somatic cell nuclear transfer up to 5-fold in a concentration- and time-dependent manner [51, 52].

Researchers speculated that reprogramming using defined reprogramming factors may follow a similar mechanism of action to that of reprogramming using somatic cell nuclear transfer. By using an Oct4-GFP reporter gene, scientists determined whether small molecules involved in chromatin modification, such as HDAC inhibitors, played a role in reprogramming mouse embryonic fibroblasts to iPSCs

[48]. Indeed, HDAC inhibitors, including suberoylanilide hydroxamic (SAHA), TSA, and valproic acid (VPA), all increased the efficiency of reprogramming greatly (Figure 2) [47, 48]. Among these HDAC inhibitors, VPA exhibited the most potent effect and enhanced the reprogramming efficiency more than 100-fold [48]. It is still unclear why VPA effect on reprogramming efficiency is significantly stronger. Suggested ideas include that the toxicity of the other chemicals at tested concentrations may be higher or that VPA may have roles beyond HDAC inhibition [48]. In addition to improving the efficiency of reprogramming four factor-transduced mouse embryonic fibroblasts, VPA also improved the efficiency of iPSC colony formation dramatically even without transduced Klf4 and c-Myc [48]. The effect of VPA on reprogramming efficiency suggests that VPA may influence a crucial step in turning somatic cells into iPSCs. Using the information found, we can infer that chromatin modifications, specifically histone acetylation status, play a critical role in reprogramming and that HDAC inhibitors can significantly improve reprogramming efficiency [48]. However, it is worth noting that many HDAC inhibitors identified so far

have low specificity [53, 54]. Moreover, each HDAC may be involved in multiple pathways. Therefore, it is important to search for more specific HDAC inhibitors and to understand the modes of action of each HDAC and HDAC inhibitor, in order to account for any unexpected side effects [53].

Recently, a simple and nonintegrating method for reprogramming has been developed by using synthetic mRNAs of the reprogramming factors, Oct4, Sox2, Klf4, c-Myc, and Lin28 [55]. This method allowed up to 36-fold increase of reprogramming efficiency, compared to the retroviral approach of delivering reprogramming factors [55]. More recently, it has been shown that the expression of the microRNA cluster miR-302/367 allowed rapid and efficient reprogramming of both mouse and human somatic cells to iPSCs without exogenous transcription factors [56]. The development of transgene-free iPSCs with high efficiency may allow patient-specific regenerative medicine within grasp.

4. Conclusions

An emerging regulatory network controlling neural stem cell self-renewal and differentiation is defined by integration of epigenetic regulators with other cell-intrinsic regulators and cell-extrinsic signals from stem cell niches. Unraveling how HDACs function within this network to regulate neural stem cell self-renewal and neurogenesis is essential to better understand neural stem cell biology. It will facilitate the development of new and targeted therapies using neural stem cells for a host of neurological disorders, including neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, and brain injuries.

The success in iPSC derivation has brought the realization of the therapeutic potential of stem cell technology closer than ever to us. However, to reach the full potential of iPSC application, it will be essential to improve the methods for iPSC generation by avoiding viral integration and enhancing the reprogramming efficiency. The recent achievement in enhancing reprogramming efficiency using HDAC inhibitors may build a foundation for future studies by allowing scientists to delve deeper into understanding the complexity of epigenetics in reprogramming and to develop even more strategies to further improve reprogramming efficiency. With the ability to generate iPSCs with increased efficiency, a huge step is taken in the branch of regenerative medicine, in addition to disease modeling, and drug development. There is no doubt that iPSC technology will have a positive impact on stem cell therapies in the future.

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Review Article

Therapeutic Strategies to Enhance the Anticancer Efficacy of Histone Deacetylase Inhibitors

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Histone acetylation is a posttranslational modification that plays a role in regulating gene expression. More recently, other nonhistone proteins have been identified to be acetylated which can regulate their function, stability, localization, or interaction with other molecules. Modulating acetylation with histone deacetylase inhibitors (HDACi) has been validated to have anticancer effects in preclinical and clinical cancer models. This has led to development and approval of the first HDACi, vorinostat, for the treatment of cutaneous T cell lymphoma. However, to date, targeting acetylation with HDACi as a monotherapy has shown modest activity against other cancers. To improve their efficacy, HDACi have been paired with other antitumor agents. Here, we discuss several combination therapies, highlighting various epigenetic drugs, ROS-generating agents, proteasome inhibitors, and DNA-damaging compounds that together may provide a therapeutic advantage over single-agent strategies.

1. Introduction

Over time, an appreciation of the importance and complexity of epigenetic events, such as DNA methylation, histone posttranslational modifications, and miRNA regulation, has fueled interest in many new areas of research. Histone acetylation is one process that is being intensely studied due to its ability to regulate gene transcription. The enzymes that regulate histone acetylation are often inappropriately expressed in cancer cells, which can lead to the silencing of tumor suppressor genes or activation of oncogenes. Because of this, many of these enzymes have become popular targets for cancer therapy. In this paper we will highlight histone deacetylase inhibitors, a group of compounds that blocks the zinc-dependent histone deacetylases involved in removing acetyl groups from lysine residues. Modulation of protein acetylation by the first-in-class FDA (U S Food and Drug Administration) approved HDACi, vorinostat, has

been shown to be successful for the treatment of refractory cutaneous T cell lymphoma (CTCL). However, despite the promising results employing HDACi as an epigenetic targeted therapy, its limited success in specific cancers as a single drug has prompted further investigation of combining HDACi with other anticancer agents. These combination regimens, which will be the focus of this review, may enhance the clinical efficacy of HDACi and may provide a therapeutic advantage in cancers where HDACi alone have limited activity.

2. Histone Deacetylases (HDACs) and Cancer

Histone deacetylases (HDACs) are a group of enzymes that, in conjunction with histone acetyltransferases (HATs), regulate the acetylation status of histone tails. HATs acetylate lysine residues on histone tails resulting in neutralization of their charge and decreased affinity for DNA [1]. This

“loosening” of the histone-DNA interaction is associated with conformational changes which allow for transcription factors to bind to the DNA and impact gene transcription [2]. HDACs, on the other hand, remove acetyl groups which lead to a more compact chromatin conformation that is often associated with gene repression. Importantly, HDACs usually do not function alone, but are part of multiprotein complexes that contain DNA-binding proteins, chromatin-remodeling proteins, and other histone-modifying proteins that participate together to regulate transcription. In addition, according to the “histone code” hypothesis, histone modifications work together with other epigenetic modifications to determine certain transcriptional outcomes [3].

HDACs are categorized into four families, class I, II, III, and IV, based on their structure. Class I, which includes HDAC 1, 2, 3, and 8, is predominately localized to the nucleus. Class II consists of HDACs 4, 5, 6, 7, 9, and 10 and is detected in both the nucleus and cytoplasm. HDAC 11 is the sole class IV member and resides in the nucleus [4]. These three classes of HDACs are zinc-dependent enzymes and are the molecular targets of HDACi. In contrast, class III is comprised of the NAD-dependent deacetylases, sirtuins (SIRT 1–7), which are found in the nucleus, cytoplasm, and mitochondria and have been identified to be involved in metabolism and aging [5]. However, they will not be discussed in this paper since they are not targets of HDACi.

There are numerous studies demonstrating that histones are not the only substrates for HDACs and HATs. These enzymes also regulate acetylation of nonhistone proteins, including transcription factors, chaperone proteins, and signaling molecules involved in cancer development and progression such as the tumor suppressor p53 [6]. In general, acetylation can interfere with binding, function, and/or stability (turnover) of the protein. Since HDACs are involved in deacetylating a wide variety of substrates they have been identified to modulate many cellular processes and thus may be used by cancer cells for a survival advantage. Based on this rationale, efforts to define which HDACs are involved in cancer development and progression are being undertaken. Many of these studies have employed HDACi to demonstrate the validity of HDACs as therapeutic targets, but effects were selective to cancer type or were inhibitor specific. However, strategies using small interfering RNA (siRNA) against class I and II HDACs have been used to determine which HDACs play a role in proliferation and survival of cancer cells. Silencing of HDAC 1 and 3 by siRNA resulted in antiproliferative effects in human cervical carcinoma cells (HeLa) [7]. However, silencing class II HDACs, HDAC 4 and 7, did not have an effect on proliferation [7]. Additionally, HDAC 3 knockdown by siRNA resulted in hyperacetylation of histone-H3 and an increase in apoptotic cell death [7]. These results suggest that, at least in the case of cervical carcinoma, class I HDACs may be better candidates for inhibition over class II isoforms. Yet, it is difficult to pinpoint which HDACs are suitable targets since examination of HDAC expression levels differs greatly from cancer to cancer, with many displaying aberrant levels. In some cases, even the surrounding normal tissue may also express high levels of HDACs, begging the question of how only malignant

cells can be targeted with HDACi and not healthy cells. Surprisingly, studies show that HDACi selectively target tumor cells at doses that have very little effect on normal cells [8–10]. This susceptibility of transformed cells to HDACi therapy is probably due to their dependence on HDACs for modulating expression of genes involved in tumor cell growth, differentiation, and apoptosis that provide cancer cells with a survival advantage (further discussed Section 4) [11]. Because of this reliance, interfering with HDACs for a therapeutic advantage in cancer is gaining momentum.

3. Development of Histone Deacetylase Inhibitors (HDACi) for Cancer Therapy

Initially, HDACi were identified by several groups as agents that induced differentiation of murine erythroleukemia cells (MELC). Transfection experiments in MELC performed by Friend et al. revealed that treatment with dimethyl sulfoxide (DMSO) resulted in synthesis and accumulation of hemoglobin, an indication of erythroid differentiation [12]. Similar results were also observed by Yoshida et al. in experiments that demonstrated differentiation of MELC with a naturally derived antifungal antibiotic, trichostatin A (TSA) [13]. Further analysis of TSA in rat fibroblasts showed that this compound induced G1 and G2 cell cycle arrest but, most importantly, subsequent studies analyzing histone modifications identified histone deacetylases as the molecular targets for TSA. During this time, Paul Marks’ group discovered that HMBA, a small molecule polar compound, was able to induce differentiation similarly to DMSO [14]. Compounds that share certain structural features with DMSO have now been synthesized in an attempt to generate compounds with increased anticancer efficacy [15]. One of these compounds is suberoylanilide hydroxamic acid (SAHA), now known as vorinostat. However, the targets of these differentiating compounds were still unknown until closer examination of the structure of vorinostat revealed a similarity to the structure of TSA. Based on these observations, Marks’ group reported in 1998 that vorinostat targeted HDACs, inhibiting HDAC 1 and 3 resulting in acetylation of histones in various cancer cell lines [15]. Subsequent studies over the next eight years demonstrated that vorinostat modulated transcription of gene expression and had antitumor selectivity in *in vivo* cancer models. Eventually, this led to its evaluation in clinical trials and the FDA approval of the first-in-class HDACi for cancer treatment.

Since these events, several HDACi have been identified either through synthetic or natural sources. HDACi can be separated into several structurally distinct classes: short-chain fatty acids (i.e., valproic acid), hydroxamic acids (i.e., vorinostat, TSA, and PCI-24781), benzamides (i.e., entinostat), cyclic tetrapeptides (i.e., depsipeptide), and electrophilic ketones. Some of these compounds selectively block specific classes of HDACs, while some have a broader spectrum of activity and therefore inhibit several classes of HDACs. For example, the benzamide entinostat (previously known as SNDX-275 or MS-275) is selective for class I HDACs (HDAC 1, 2, and 3), while the HDACi tubacin specifically targets

only HDAC 6 [16]. In contrast, the majority of hydroxamic acids, including vorinostat, panobinostat (LBH589), and TSA, behave as pan-HDACi, blocking several classes of HDACs, although more selective hydroxamic acid inhibitors are being developed. For example, a novel hydroxamic acid, PCI-24781, is currently in phase I clinical trials, preferentially inhibits class I and II HDACs, and is more potent at targeting these isoforms compared to vorinostat [17].

4. HDACi: Mechanisms of Action

Treatment with HDACi has been found to change the gene expression of about 7% of the genes studied, indicating that HDACi can be used to alter a subset of genes [18]. In fact, gene expression analyses have demonstrated that HDACi can selectively induce apoptosis in cancer cells by upregulating and/or downregulating the expression of proapoptotic and antiapoptotic genes [19]. Mechanistic studies have implicated activation of the death-receptor (extrinsic) pathway or the mitochondrial (intrinsic) apoptotic pathways as a mechanism of action of different HDACi. Induction of distinct cell death pathways may be associated with the structurally diverse HDACi, which have different targets and have been demonstrated to be cell-type dependent.

The role of HDACi in triggering the extrinsic apoptotic pathway has been demonstrated by several *in vitro* studies. In these experiments, HDACi have shown to activate death receptors, including TRAIL, DR5, FAS, and TNF alpha [19]. These observations have been validated by studies where the inhibition of death receptors and their ligands abrogated HDACi-dependent apoptosis [10, 20]. In addition, *in vivo* experiments suppressing TRAIL and Fas by siRNA in mice resulted in a significant reduction in apoptosis after treatment with the HDACi, valproic acid [21]. Yet, several studies have also implicated the involvement of the intrinsic pathway in HDACi-induced apoptosis. HDACi can transcriptionally regulate the expression of proapoptotic BH3-only proteins including Bid, Bad, and Bim, which play an important role in the activation of the intrinsic apoptotic pathway [22–24]. Furthermore, elevated levels of reactive oxygen species (ROS) have been observed after treatment with HDACi [24–26]. The increase in ROS has been shown to precede changes in mitochondrial membrane potential [27], suggesting a link between HDACi, ROS, BH3-only proteins and activation of the intrinsic pathway.

In fact, oxidative stress has been identified as a mechanism by which HDACi may be exerting its lethal effects in tumor cells. However, the manner by which HDACi induce oxidative stress is not well understood. Two prominent mechanisms have been reported. One involves mitochondrial injury, while the other implicates modulating antioxidants levels (Figure 1). In an acute leukemia treated cell line, vorinostat induced apoptosis by the expression of proapoptotic Bid, which resulted in disruption of mitochondria, a major source of ROS in the cell. Accordingly, subsequent production of ROS was observed in these cells (Figure 1(a)) [24]. More recently, detailed studies by Paul Marks' laboratory have demonstrated that vorinostat and entinostat upregulate

thioredoxin binding protein-2 (TBP-2), which is a protein that binds and inhibits thioredoxin (Trx) (Figure 1(b)) [28]. Trx is a ubiquitous protein with pleiotropic effects, with one of its major functions to operate as an intracellular antioxidant. Interestingly, studies have shown that this antioxidant is upregulated in certain types of tumors [29, 30] perhaps giving cancer cells a survival advantage to deal with the elevated oxidative stress. These findings indicate that Trx may be a good candidate to target for the treatment of cancer. Ungerstedt and colleagues demonstrated that exposure of transformed cells to HDACi resulted in ROS-dependent apoptosis. Furthermore, nontransformed cells were resistant to this HDACi treatment; instead an increase in Trx levels was detected, and no production of ROS was observed. The rise of HDACi-induced Trx expression in nonmalignant cells offered cytoprotection since siRNA against the antioxidant resulted in increased oxidative stress and sensitivity towards HDACi [9]. These observations of Trx overexpression offering a protective mechanism against HDACi provides an additional explanation of the selectivity of HDACi for some cancer cells compared to nontransformed cells.

HDACi have also been shown to induce cell cycle arrest. The mechanism by which HDACi induce cell cycle arrest includes the induction of cell cycle genes like CDKN1A which encode the production of p21^{WAF1/CIP1} [22, 31]. Also, HDACi can transcriptionally repress cyclin D and cyclin A genes resulting in the loss of CDK2 and CDK4 kinase activity [32, 33]. In addition to the induction of apoptosis and cell cycle arrest, HDACi have been shown to have antiangiogenic effects by downregulating proangiogenic genes like vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) [34]. These antiangiogenic effects have been observed in different cancer models both *in vitro* and *in vivo* [34–36]. These studies support the possibility of HDACi interfering with the metastatic process. However, more studies are needed to understand better their role in metastasis.

5. HDACi in the Clinic

Preclinical studies of HDACi, in cell lines and animal models, have proven to be very successful as single-modality agents for the treatment of a variety of cancers. As a result, several structurally different HDACi have been used in hundreds of clinical trials to test their toxicity and efficacy. In general, clinical trials involving HDACi alone, or in combination with other chemotherapeutic agents, yield promising results and demonstrate biological and antitumor activity.

Vorinostat is the first HDACi to show promise in the clinic. In phase I and II trials, vorinostat was well tolerated and ~30% of CTCL patients enrolled in the study received clinical benefit [37]. However, in other phase II trials evaluating the efficacy of vorinostat in solid tumors, including ovarian [38], breast, colorectal, nonsmall cell lung [39], head and neck [40], and glioblastoma [41], only a moderate effect was observed. Moreover, treatment of metastatic tumors with vorinostat had limited success [40, 42, 43].

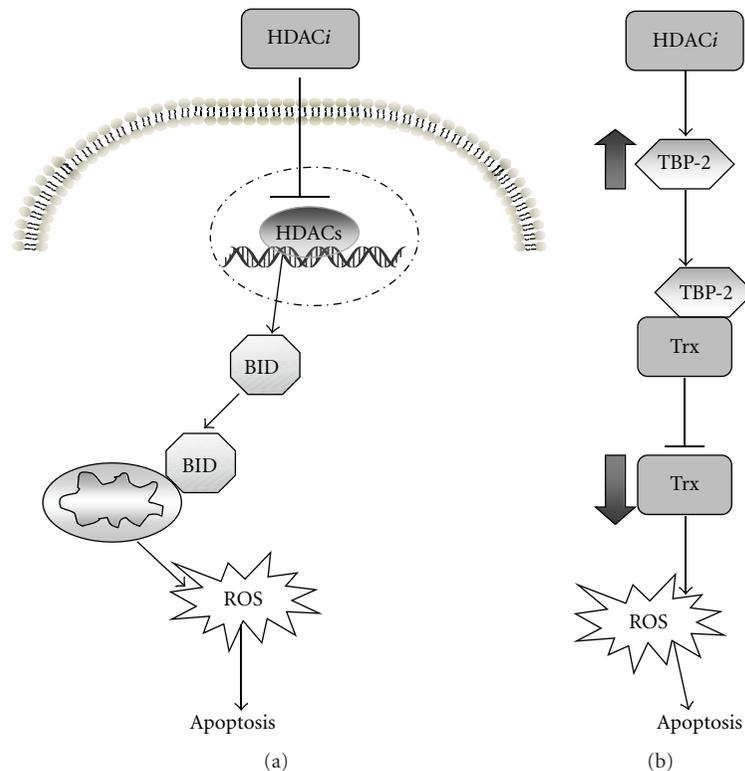


FIGURE 1: Proposed mechanisms by which HDACi induce ROS. (a) Mitochondrial injury. HDACi induces expression of Bid protein. This proapoptotic molecule binds to and disrupts the mitochondrial membrane, which results in increased ROS levels and apoptosis. (b) Alterations in antioxidant levels. HDACi upregulate the expression of thioredoxin-binding protein-2 (TBP-2). TBP-2 binds to and inhibits the antioxidant thioredoxin (Trx). This inhibition results in an imbalance of oxidants and antioxidants leading to increased ROS levels, which promotes apoptosis.

Upon the success of vorinostat in CTCL and its approval by the FDA for this disease, several new HDACi were developed and have been investigated in clinical trials (reviewed in [44]). There are currently close to 50 active clinical trials evaluating several HDACi as monotherapies for a variety of cancers and over 100 trials combining HDACi with other chemotherapeutic agents (<http://www.clinicaltrials.gov/>, <http://www.cancer.gov/clinicaltrials>). These studies include pan-HDACi, such as the novel compound PCI-24781 (Pharmacocyclics), as well as isoform-specific HDACi, such as entinostat. Preclinical studies using PCI-24781 demonstrate an inhibition in cell growth and an increase in apoptosis, and treatment of colon tumor xenografts significantly reduces tumor volume [17]. PCI-24781 is currently under evaluation in phase I trials for sarcoma, non-Hodgkin lymphoma, multiple myeloma, and chronic lymphocytic leukemia. Another structurally different HDACi, entinostat, is more selective for class I HDACs. Like vorinostat, entinostat shows the greatest therapeutic response in patients with leukemia and lymphoma [45, 46] whereas it is only moderately effective for solid tumors [47, 48]. Entinostat is currently being evaluated as mono- and combination therapies for a variety of cancer types (Table 1).

Despite promising preclinical *in vitro* and *in vivo* data evaluating HDACi, clinical trials using these agents as monotherapies have mostly been successful in treating CTCL

and hematological malignancies. One explanation for this observation is the inability to achieve appropriate doses of HDACi and consistent acetylation of target proteins. *In vitro* studies evaluating HDACi require at least 24 hours and micromolar concentrations to cause tumor cell death. Phase I clinical trials of vorinostat demonstrate that plasma concentrations (C_{max}) reach the micromolar range, $2.5 \mu\text{M}$ for oral administration of 400 mg/d and $9 \mu\text{M}$ for 300 mg/m² per day for intravenous administration [49]; however the half life is relatively short, 91.6–127 minutes orally and 34.7–42.4 minutes intravenously [50]. These data suggest that continuous administration of these agents may be necessary to achieve clinical response. This may not be feasible with a broad-spectrum HDACi, like vorinostat, due to the large number of acetylated target proteins which may contribute to dose-limiting toxicities. HDACi clinical trials primarily focus on evaluating the acetylation status of histone H3 and H4 to determine whether these compounds are blocking their substrates. These pharmacodynamic studies have revealed that increased acetylation of histone H3 and H4 is observed in peripheral blood mononuclear cells (PMBCs) and/or bone marrow mononuclear cells (BMMCs) of patients treated with HDACi, indicating that these inhibitors are targeting HDACs. However, similar increased acetylation has been detected in both responders and nonresponders suggesting that increased histone acetylation in PMBCs and BMMCs

TABLE 1: HDACi combination regimens currently in clinical trials. Partial list of clinical trials evaluating the combination of HDACi with other anticancer agents discussed in this paper (source: <http://www.clinicaltrials.gov/>, <http://www.cancer.gov/clinicaltrials>).

HDACi	Other Intervention	Tumor Type
Entinostat (SNDX-275)	azacitidine	leukemia, MDS, colorectal cancer, NSCLC
Mocetinostat (MGCD0103)	azacitidine	MDS
Panobinostat (LBH589)	azacitidine	MDS, CML, AML
	bortezomib	peripheral T cell lymphoma, NK/T cell lymphoma, MM, pancreatic cancer
	bortezomib, dexamethasone	MM
	decitabine	MDS, AML
	decitabine, temozolomide	melanoma
	radiation therapy	prostate cancer, esophageal cancer, head and neck cancer
PCI-24781	doxorubicin	sarcoma
Romidepsin	bortezomib	myeloma
Valproic acid	azacitidine	advanced cancers
	azacitidine, ATRA	AML, MDS
	decitabine, ATRA	AML
	radiation therapy, bevacizumab	children with high grade gliomas
	radiation therapy, temozolomide	high grade gliomas, brain tumors
Vorinostat	azacitidine	AML, MDS, DLBCL, lymphoma, MM, NSCLC, Non-Hodgkin's lymphoma
	bortezomib, AMG 655	lymphoma
	bortezomib, dexamethasone	MM
	cisplatin, pemetrexed, radiation therapy	NSCLC
	cisplatin, radiation therapy	squamous cell carcinoma
	gemtuzumab ozogamicin, azacitidine	AML
	marizomib (NPI-0052)	NSCLC, pancreatic cancer, melanoma, lymphoma
	paclitaxel, radiation therapy	NSCLC
	radiation therapy	brain metastases, NSCLC, pancreatic cancer, pelvic cancer
	radiation therapy, fluorouracil	pancreatic adenocarcinoma

ATRA: all-trans retinoic acid, MM: multiple myeloma, NSCLC: nonsmall cell lung carcinoma, AML: acute myeloid leukemia, CML: chronic myelogenous leukemia, MDS: myelodysplastic syndrome, and DLBCL: Diffuse large B-cell lymphoma.

does not correlate with clinical response. Histones are not the only proteins capable of being acetylated. Our knowledge of acetylated nonhistone proteins is rapidly increasing through efforts to define the acetylome [51] but the biological relevance of acetylation on many of these proteins is still mostly unknown. A greater understanding of the acetylome may reveal other molecular endpoints that might indicate a favorable clinical response or that might identify contributors of dose-limiting toxicities. In addition, deciphering the individual role of each individual HDAC in cancer progression will aid in knowing which specific isoform to target. However, even isoform-specific inhibitors may not completely eliminate on-target dose-limiting toxicities because most HDACs reside in multiple large multiprotein complexes. A single HDAC can simultaneously play different roles within the cell depending on which complex it is associated. In addition to the specificity of HDACi, the lack of response of some

patients to HDACi therapy may be attributed to mechanisms of resistance [50].

Overall, HDACi have shown promise in the clinic but there is clearly room for improvement of therapeutic index. One way to achieve greater clinical efficacy is to use HDACi in combination with other chemotherapeutic agents. In fact, many preclinical studies provide evidence supporting synergistic or additive effects of HDACi in combination with other cytotoxic agents, and a partial list of HDACi combination trials is found in Table 1. The agents listed will be the focus of the remainder of the paper.

6. Combining HDACi with Other Epigenetic Therapies

The predominant function of HDACs is the modification of histone tails which influences gene transcription. In addition

to histone acetylation, gene transcription is also controlled by DNA methylation and histone methylation. These processes often work in concert with one another providing the rationale for combining epigenetic therapies for cancer treatment.

6.1. DNA Methyltransferases. DNA methylation involves the covalent addition of a methyl group to cytosine residues of DNA by enzymes termed DNA methyltransferases (DNMTs). Often, DNA methylation occurs within CpG islands located within the 5' promoter regions of genes [52]. DNA methylation can inhibit transcription directly by influencing transcription factor binding [53] but also maintains chromatin in a transcriptionally inactive state through the recruitment of methyl-CpG binding proteins (MBDs) [54, 55], some of which recruit histone deacetylases for added epigenetic control [56, 57]. In normal cells, the CpG islands of transcriptionally active genes are not methylated [52]. However, in cancer, many of the unmethylated genes become aberrantly methylated [58–60]. The discovery that CpG methylation was a causative event in tumor progression led to the search for drugs which could reverse the DNA methylation and restore gene expression.

The first-FDA approved DNMT inhibitors (DNMTi) are the nucleoside analogs 5-azacytidine (azacitidine) and 5-aza-2'-deoxycytidine (decitabine). Nucleoside analogs, as well as nonnucleoside analog DNMTi, are effective anticancer agents that cause increased apoptosis [61]. Moreover, these agents reverse the DNA hypermethylation association with certain cancer genes and alter gene expression [61–63]. However, there is a wide variation among the different agents which led to the hypothesis that DNA methylation influences the stability of other chromatin marks prompting the evaluation of combined use of DNMTi and HDACi [64].

Several preclinical studies evaluating the effects of DNMTi in combination with HDACi demonstrate synergistic anticancer activity. For example, cotreatment of prostate cancer cells [65] or pancreatic cancer cells [66] with decitabine and TSA led to reduced cell proliferation which was accompanied by increased apoptosis. Similar results were obtained in other cell lines where enhanced apoptosis was observed in AML1/ETO-positive acute myelogenous leukemia (AML) cells [67] and decreased cell proliferation was observed in lung cancer cells [68] treated with depsipeptide and decitabine. Moreover, entinostat and azacitidine display synergistic cytotoxicity and apoptosis in leukemia cells which correlates with enhanced rates of histone acetylation as well as elevated intracellular reactive oxygen species [26]. Given the promising preclinical data combining HDACi with DNMTi, several clinical trials utilizing the combination were administered to patients with hematologic and solid tumors where many of the patients had minimal side effects and some achieved complete and partial remissions [69, 70]. Currently, there are 14 trials evaluating DNMTi with HDACi (Table 1).

6.2. Histone Demethylases. The methylation status of histones also plays an important role in gene expression. Although, for many years, histone methylation was considered

to be a stable, irreversible modification, recently, two families of enzymes have been discovered which function to remove methyl groups from the lysines of histone and nonhistone proteins. The first enzyme to be discovered was the lysine-specific demethylase 1 (LSD1) which functions similar to the amine oxidase family of enzymes [71]. The second family of enzymes discovered are the jumonji-domain-containing proteins [72]. These metalloenzymes mediate hydroxylation-based demethylation of lysines [72]. Both of these families of demethylase enzymes have been reported to reside in complexes containing HDACs [73, 74], and the activity of LSD1 is influenced by HDAC function [74], providing the rationale for targeting both enzymes as epigenetic therapy.

Due to the structural similarity between LSD1 and the amine oxidase family of enzymes, several groups have demonstrated that mono- and polyamine oxidase inhibitors also target LSD1 [74–79]. Studies from our laboratory evaluating cotreatment of glioblastoma cells with the combination of HDACi, vorinostat or PCI-24781, with the LSD1 inhibitor, tranlycypromine, show a synergistic increase in apoptotic cell death [80]. Moreover, treatment of normal human astrocytes with the same doses of HDACi and tranlycypromine did not yield enhanced cell death suggesting that the synergistic apoptosis induced by the combination is selective for glioblastoma cells [80]. These data support the use of HDACi and LSD1 as combination therapy in preclinical mouse studies. In addition, future studies aimed at understanding the molecular mechanisms by which HDACs and LSD1 regulate cancer cell growth have the potential to identify new molecular targets for therapy.

Not only there is cross-talk between LSD1 and HDACs, but also LSD1 is required for the maintenance of global DNA methylation [81]. Moreover, LSD2, a homolog of LSD1, participates in establishing maternal genomic imprints during oogenesis [82]. These data suggest that targeting the LSD family and DNMTs may enhance the antitumor activity of these drugs. In fact, inhibition of LSD1 in human colorectal cells with a combination of novel oligoamine analogs and DNMTi led to a greater re-expression of aberrantly silenced genes when compared to either agent used alone [83]. In addition, colorectal xenograft models treated with the combination of PG-11144, an oligoamine analog inhibitor, and azacitidine caused dramatic decreases in tumor cell growth demonstrating the therapeutic efficacy of this combination [83].

The discovery of lysine demethylases and the influence these enzymes have on many biological processes has led to the recognition of their potential as a therapeutic target in a variety of diseases, including cancer. Developing more specific inhibitors for the demethylase enzymes, particularly the jumonji-domain-containing family where investigators are just now starting to identify inhibitors [84], will aid in understanding what role individual demethylases play during cell growth and development. These studies will make substantial contributions to our knowledge regarding epigenetic regulators and are needed to utilize epigenetic therapies to their fullest potential.

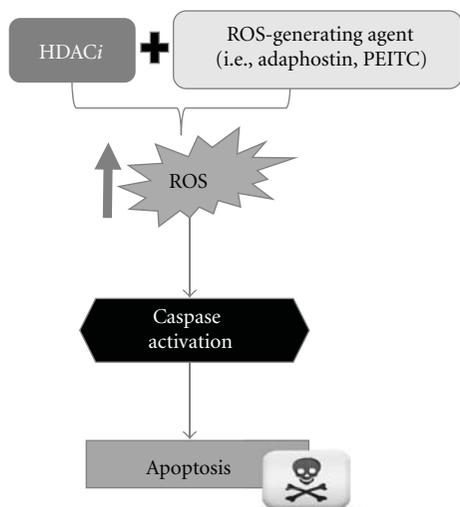


FIGURE 2: Activation of apoptosis by combinations of HDACi and ROS-generating agents. HDACi combined with agents like adaphostin and PEITC result in an increase production of ROS in cancer cells. The high levels of ROS result in induction of apoptosis via caspase activation.

7. HDACi and ROS-Generating Agents

Several reports have shown that HDACi induce oxidative stress in different types of cancer cells [85]. Cancer cells also have higher levels of reactive oxygen species (ROS) compared to normal cells, most likely as a consequence of an active metabolism and more robust proliferation rates [86, 87]. This difference has been used as a therapeutic strategy to treat cancer. Combining HDACi with agents that cause further oxidative stress might enhance the efficacy of HDACi for the treatment of cancer (Figure 2).

7.1. Adaphostin. Adaphostin is a drug that is part of the tyrosinostatin family of tyrosine kinase inhibitors, and it affects a number of different kinases. It is an analog of AG957, a drug that was originally developed to inhibit p210^{Bcr/abl} [88]. However, studies have demonstrated that adaphostin's activity is not restricted by the presence or absence of Bcr/Abl kinase [89]. This compound has been identified as a potential anticancer agent to treat acute leukemias such as AML and ALL (acute lymphoblastic leukemia). Importantly, adaphostin demonstrates selectivity for leukemia cells as compared to normal lymphocytes [85]. Mechanistic studies have demonstrated that adaphostin elevates levels of intracellular ROS, resulting in apoptosis [90]. Additional investigations by Le et al. expand these observations by showing that the increase in ROS in cells treated with adaphostin is the result of its accumulation in the mitochondria, where adaphostin binds to complex III, inhibiting electron transport [91] and leading to oxidative stress. Moreover, transcriptional and proteomic analyses of adaphostin-treated cells demonstrated an upregulation of oxidative stress-related genes and antioxidants [92], including the genes encoding heat shock proteins, glutathione S-transferase (GST), and

superoxide dismutase. A decrease in the antioxidant glutathione has also been observed in studies in CML (chronic myelogenous leukemia) cells treated with adaphostin [90]. Taken together, these observations indicate that adaphostin is a redox-modulatory agent and a good candidate to combine with HDACi.

Unpublished data from our group shows strong synergy between two structurally different HDACi (entinostat and vorinostat) and adaphostin resulting in apoptosis in leukemia cells. Results showed a threefold increase in DNA fragmentation, a hallmark of apoptosis, when cells were treated with adaphostin combined with entinostat compared to cells treated with HDACi alone. A more potent effect was achieved with adaphostin and vorinostat, demonstrating a sixfold increase in DNA fragmentation. Furthermore, these combinations enhanced superoxide levels, suggesting that oxidative stress plays a role in the synergistic induction of apoptosis. The results observed with these combinations lend support to the idea of enhancing the efficacy of HDACi by modulating ROS levels with an oxidant-generating agent that may push the balance towards oxidative stress and cell death and provide a therapeutic advantage for the treatment of cancers such as leukemia.

7.2. β -Phenylethyl Isothiocyanate (PEITC). A second redox-modulatory agent, which has shown promises for the treatment and prevention of cancer, is PEITC. This agent is a natural compound found in cruciferous vegetables like cauliflower, broccoli, and cabbage. PEITC has been shown to be effective in cancer cells by inhibiting carcinogenesis and inducing cell growth arrest and apoptosis [93]. Studies in prostate cancer cells demonstrate that PEITC induce apoptosis by decreasing the levels of the antiapoptotic proteins Bcl-2 and Bcl-X_L [94]. In addition, it has been shown to downregulate and facilitate the degradation of the androgen receptor [95]. More detailed mechanistic studies have revealed the main mechanism by which PEITC works as an anticancer agent is through redox-modulating mechanisms. These mechanisms include the inhibition of cytochrome P450 and the induction of metabolizing enzymes like NAD(P)H: quinone oxidoreductase (NQO-1) and GST [93]. A more recent study reports production of ROS by PEITC to be mediated by inhibition of complex III and oxidative phosphorylation [96]. In addition, *in vitro* and *in vivo* studies have demonstrated PEITC to induce an accumulation in ROS that is mediated by the depletion of the antioxidant, glutathione (GSH) [97]. Furthermore, the increase of ROS and depletion of GSH by this agent have been shown to overcome the resistance of leukemia cells to fludarabine [98].

Taking into consideration that PEITC is a ROS modulating agent, it can perhaps be combined with HDACi to improve its efficacy. Recently, Hu et al. addressed this assumption and demonstrated that depletion of GSH by PEITC increases sensitivity to vorinostat in leukemia cells. Furthermore, the combination of HDACi/PEITC also induced ROS accumulation and apoptosis in a vorinostat-resistant cell line via activation of the NADPH oxidase. Induction of ROS by this combination allows for the translocation of transcription

factor Nrf2 to the nucleus, stimulating transcription of genes involved in the glutathione system [99].

All together, these studies suggest that the regulation of oxidative stress plays an important role in the cytotoxic effects with HDACi and ROS generating agents. Further understanding how HDACi, alone or in combination with other redox-modulating agents, regulate oxidative stress will help in the development of better therapeutic strategies for clinical utility. Presently there are multiple clinical trials combining HDACi with other chemotherapies that together have been demonstrated to increase ROS. Some of these agents include proteasome inhibitors and DNA-damaging agents. The combination of HDACi with these agents will be discussed in the next two sections.

8. Proteasome Inhibitors and HDACi Regimens

A growing body of work in the literature is providing evidence to support the use of proteasome inhibitors as an option to combine with HDACi. The molecular targets of these compounds are the enzymatic activities housed within the proteolytic chamber of the proteasome. These include the chymotrypsin-, caspase-, and trypsin-like proteolytic activities that are responsible for degrading the majority of intracellular proteins [100]. Thus, inhibiting the proteasome will influence many signaling pathways and cellular processes, including cell growth and survival, tumor suppression, and apoptosis. Surprisingly, most likely due to their rapid protein turnover rate, cancer cells rely heavily on the proteasome to dispose of unwanted proteins and therefore are more susceptible to proteasome inhibition compared to nontransformed cells [101, 102]. One of the most commonly reported consequences of proteasome inhibition in tumor cells is cell death. As a result of this selectivity, similar to HDACi, compounds that target the proteasome have emerged as novel cancer therapies in the recent years. However, despite the promise of both HDACi and proteasome inhibitors in preclinical and *in vivo* models as single agents, similar responses have not been duplicated in clinical settings. One way to overcome these unexpected shortcomings has been to combine these two different compounds to enhance their antitumor activity. Specifically, three clinically relevant proteasome inhibitors—bortezomib, marizomib (formerly known as NPI-0052), and carfilzomib—are proving to be strong candidates for combination regimens with HDACi.

8.1. Bortezomib. Originally synthesized as an inhibitor of the chymotrypsin-like activity of the proteasome, the boronic acid-derived compound bortezomib is the sole FDA-approved drug of its class for multiple myeloma (MM) and mantle cell lymphoma (MCL). This reversible inhibitor has been shown to work in combination with HDACi by inducing cytotoxic effects mediated primarily by cellular stress, JNK (Jun NH₂-terminal kinase) activation, and upregulation of proapoptotic proteins. Stress to the cell is a commonly characterized event produced by the combination treatment that triggers apoptosis in cancer cells, and two major sources of stress stand out (Figure 3). The first one involves

the generation of reactive oxygen species. Pretreatment with bortezomib sensitized MM cells to two different HDACi, vorinostat and sodium butyrate, inducing synergistic apoptosis [103]. Mitochondrial injury, JNK activation, caspase activation, and increased oxidative stress were among the events observed with the combination regimen. Importantly, treatment with an antioxidant markedly decreased JNK activation and apoptosis suggesting that ROS was contributing to these effects [103]. Similar ROS-dependent apoptosis was also observed in Bcr/Abl+ leukemic cells with the same combination treatment. Moreover, cell death was induced by bortezomib/HDACi in Gleevec-resistant K562 (Bcr/Abl+ CML) cells and patient-derived CD134+ cells that were refractory to Gleevec therapy [104]. These findings indicate that this proteasome inhibitor/HDACi regimen may provide benefits in cancers that have acquired resistance to their current therapies. Cytotoxic oxidative stress and DNA damage have also been reported in MM cells when bortezomib was paired with another HDACi, PXD101 [105]. Bhalla et al. report greater lethality in lymphoma cells when bortezomib was combined with PCI-24781 compared to single-agent treatment. The cell death observed in this model system was also ROS dependent [106]. Furthermore, gene expression analyses revealed downregulation of antioxidant genes with PCI-24781, and these effects were further enhanced when combined with bortezomib [106]. These results hint at a process by which oxidative stress may be altered when pairing HDACi with bortezomib. However, the source of ROS when these molecular-targeted therapies are combined is not completely clear. For proteasome inhibitors as single agents, studies in nonsmall cell lung cancer using peptide inhibitors seem to suggest that the mitochondrial electron transport chain is involved in producing oxidative stress [107]. However, these observations do not rule out involvement of other ROS-generating systems and do not reveal if it is specific to particular cancer types. For HDACi, inhibition of the antioxidant thioredoxin has been implicated for production of ROS by HDACi [28]. Further examination into the interaction between proteasome inhibitors and HDACi may reveal additional mechanisms at play, similar to those observed by Bhalla et al. in their lymphoma studies.

The other relevant mechanism that is thought to be important in the synergistic effects between these two agents is disruption of aggresome formation. Work by Nawrocki et al. showed that bortezomib induced ubiquitin-conjugated protein aggregates, which appeared to provide a protective mechanism to cells exposed to proteasome inhibitors [108]. Studies using *in vivo* and *in vitro* pancreatic cancer models demonstrated that these cytoprotective benefits were compromised when employing vorinostat or siRNA against HDAC 6 (a cytoskeleton-associated HDAC known to be required for aggresome formation), resulting in endoplasmic reticulum (ER) stress and synergistic apoptosis [108]. This was a selective effect since neither aggresome formation by bortezomib nor apoptosis by bortezomib/vorinostat regimen was observed in normal human pancreatic epithelial cells or in murine pancreatic epithelial cells *in vivo*. These observations supported earlier work in MM cells using the specific HDAC 6 inhibitor, tubacin, which synergized with

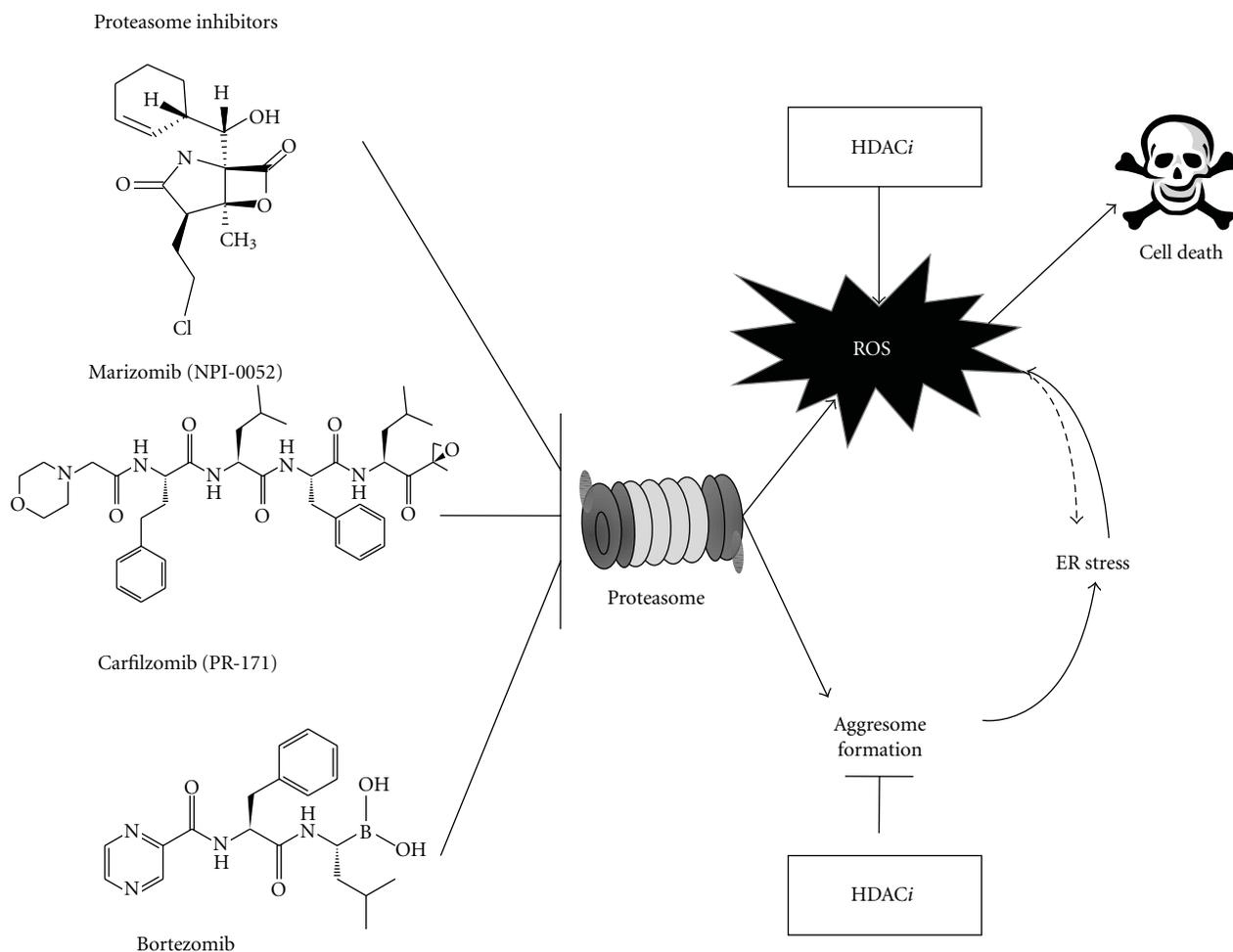


FIGURE 3: Combination of HDACi with proteasome inhibitors induces cellular stress. Synergistic apoptosis is observed between HDACi and three structurally different proteasome inhibitors—marizomib, carfilzomib, and bortezomib. The cell death observed with this therapeutic strategy is generally oxidant dependent. Individually both proteasome inhibitors and HDACi generate ROS, either via mitochondrial injury or by disregulating antioxidant systems as described in the text. When paired, these two compounds dramatically increase oxidative stress, which leads to apoptosis. High levels of ROS can also cause damage to proteins which can contribute to ER stress. Inhibiting the proteasome also results in aggregates of conjugated ubiquitin proteins that were originally to be degraded by the proteolytic complex. HDAC 6 mediates aggresome formation as a cytoprotective measure in the cell. Addition of HDACi disrupts aggresomes, leading to ER stress, which can stimulate oxidative stress or directly induce apoptosis.

bortezomib to induce lethality [109]. Both oxidative stress and interfering with aggresome formation leading to induction of ER stress are important pathways described that are considered to contribute to the synergy observed between bortezomib and HDACi. However, since both of these drugs have many pleiotropic effects, one cannot discard other mechanisms also being involved. Nevertheless, the preclinical evidence demonstrating synergy between these compounds warrants studying this combination in patients. Indeed, combination therapy between bortezomib and HDACi is currently being evaluated in numerous clinical trials (Table 1).

8.2. Marizomib. Marizomib is a clinically relevant naturally derived proteasome inhibitor that has been shown to block all three enzymatic activities of the proteasome resulting in

programmed cell death in leukemic, MM, Waldenstrom's macroglobulinemia, colorectal, and pancreatic cancer cells [110–114]. The combination of marizomib and vorinostat is being evaluated in a phase I clinical trial in patients with selected advanced malignancies (Table 1). We combined this irreversible proteasome inhibitor with HDACi (vorinostat or entinostat) and showed for the first time that this regimen induced synergistic apoptosis in both primary and cultured acute leukemia cells [115]. Isobologram analysis indicated that these synergistic effects were stronger than those achieved with a bortezomib and HDACi combination. Intracellular superoxide levels were also observed with marizomib/entinostat or vorinostat treatment compared to single agents in a Jurkat ALL cell line [115]. Work in MM and our studies in leukemia had previously identified caspase-8 as an important regulator of marizomib-induced apoptosis [110, 113]. Furthermore, we had also shown that the cytotoxicity

observed in leukemia cells with marizomib was oxidant dependent since an antioxidant prevented apoptosis. Using a variant of the Jurkat cell line that lacked caspase-8, we confirmed the requirement of this caspase for ROS-generation by marizomib alone and in combination with HDACi [115]. Interestingly, we also observed that marizomib and HDACi shared overlapping biochemical effects. Both vorinostat and entinostat were able to downregulate mRNA expression levels of beta subunits that contain the proteolytic activities of the proteasome and accordingly decreased their enzymatic effects [115]. We also showed that marizomib was able to increase histone-H3 expression and acetylation [115]. This was the first report of this epigenetic alteration, usually associated with HDAC inhibition, occurring as a consequence of proteasome inhibition, and it was specific to marizomib since bortezomib did not elicit the same effect. Furthermore, caspase-8 influenced the acetylation triggered by marizomib, since the proteasome inhibitor did not influence the acetylation in caspase-8-deficient cells, but this effect was recovered when caspase-8 was re-expressed. Overall, our results demonstrated that caspase-8 and oxidative stress contribute to the synergy observed between marizomib and HDACi. Unlike bortezomib, the novel second-generation proteasome inhibitor also had the ability to influence an epigenetic modification, as demonstrated by acetylation of histone H3, and provided another potential mechanism explaining why more synergy is observed with marizomib/HDACi compared to a bortezomib/HDACi regimen. However, a more recent study suggests that bortezomib may also be capable of modulating acetylation. In fact, their work with bortezomib may provide the missing link observed with marizomib's caspase-8-dependent acetylation. Kikuchi et al. report transcriptional downregulation of class I HDACs by bortezomib, which is mediated by caspase-8-dependent degradation of transcription factor, SP1 [116]. These data provides further evidence that proteasome inhibitors share an overlapping mechanism with HDACi.

8.3. Carfilzomib. Formerly known as PR-171, carfilzomib is a newly described irreversible proteasome inhibitor. This synthetic epoxyketone-based inhibitor is selective to potently block the chymotrypsin-like activity and is currently in clinical trials for MM and non-Hodgkin's lymphoma [117]. Compared to bortezomib, carfilzomib more potently killed MM cells and overcame resistance in patient-derived cells that demonstrated bortezomib resistance in the clinic [118]. Given that bortezomib is able to interact with HDACi to cause apoptosis, it is plausible that this next-generation proteasome inhibitor can also act synergistically with HDACi. Studies in diffuse large B cell lymphoma (DLBCL) demonstrated that carfilzomib and vorinostat interacted synergistically in DLBCL [119]. Notably, increased JNK activation contributed to lethality since interference RNA against JNK, dominant negative JNK, and peptide inhibitors reduced JNK activation and attenuated carfilzomib/HDACi cytotoxicity [119]. Combination treatment with carfilzomib and vorinostat also increased DNA damage, apoptosis and reduced tumor growth in mouse models. This regimen also showed

activity against primary cells and DLBCL cells resistant to bortezomib [119].

Oxidative stress, ER stress, and JNK activation are common pathways by which proteasome inhibitors and HDACi work in concert to enhance lethality in tumor cells. However, mechanistic studies between HDACi and the next-generation proteasome inhibitors are identifying new potential mechanisms, such as acetylation by marizomib that may also be involved in triggering cytotoxicity. Given that both HDACi and proteasome inhibitors influence numerous cellular effects, further studies examining their interactions may reveal additional overlapping mechanisms that have not yet been identified as contributing to their synergism. Together, the preclinical studies between proteasome inhibitors and the epigenetic modifiers, HDACi, have provided substantial data reinforcing the potential clinical utility of these two compounds, and results from the current clinical trials are anxiously being awaited.

9. HDACi and DNA-Damaging Agents

Another combination that may provide synergistic benefits to cancer patients is that of HDACi with agents that cause DNA damage. HDACi have been widely shown to lead to radiosensitization in cell lines and in some mouse models, and there are many possible explanations for these effects. One idea is that histone deacetylase inhibitors are capable of disrupting the DNA damage response, so their addition prolongs the effects of DNA-damaging agents. This idea is supported by the fact that many researchers have observed prolonged γ -H2AX, which indicates the presence of DNA double-strand breaks, when HDACi are added to treatment with DNA-damaging agents such as radiation [120, 121]. Another possible explanation is that treatment with HDACi "loosens" the chromatin conformation, making the DNA more accessible to damaging agents. While it is difficult to provide concrete evidence for this idea, it is definitely clear that chromatin structure is an important consideration for the cell's ability to sense and repair double-strand breaks [122]. A final idea for why HDACi are effective radiosensitizers draws from the ability of HDACi to modulate gene expression, so they may act by changing expression of genes specifically involved in DNA damage responses or survival [123]. This hypothesis is supported by previous reports that HDACi lead to increases in several well-known proapoptotic proteins, such as Bim and Bmf [123]. To test these hypotheses, many HDACi have been combined with DNA-damaging agents such as radiation and chemotherapeutic agents.

9.1. Radiation. Several HDACi have shown efficacy as radiosensitizing agents when combined with ionizing radiation (IR). Many different HDACi have been tested in a wide panel of different cell lines. Some models where HDACi have been shown to radiosensitize cell lines include the use of valproic acid in colon cancer [120] and brain tumors [124], panobinostat (LBH589) in non small cell lung cancer [121], TSA in

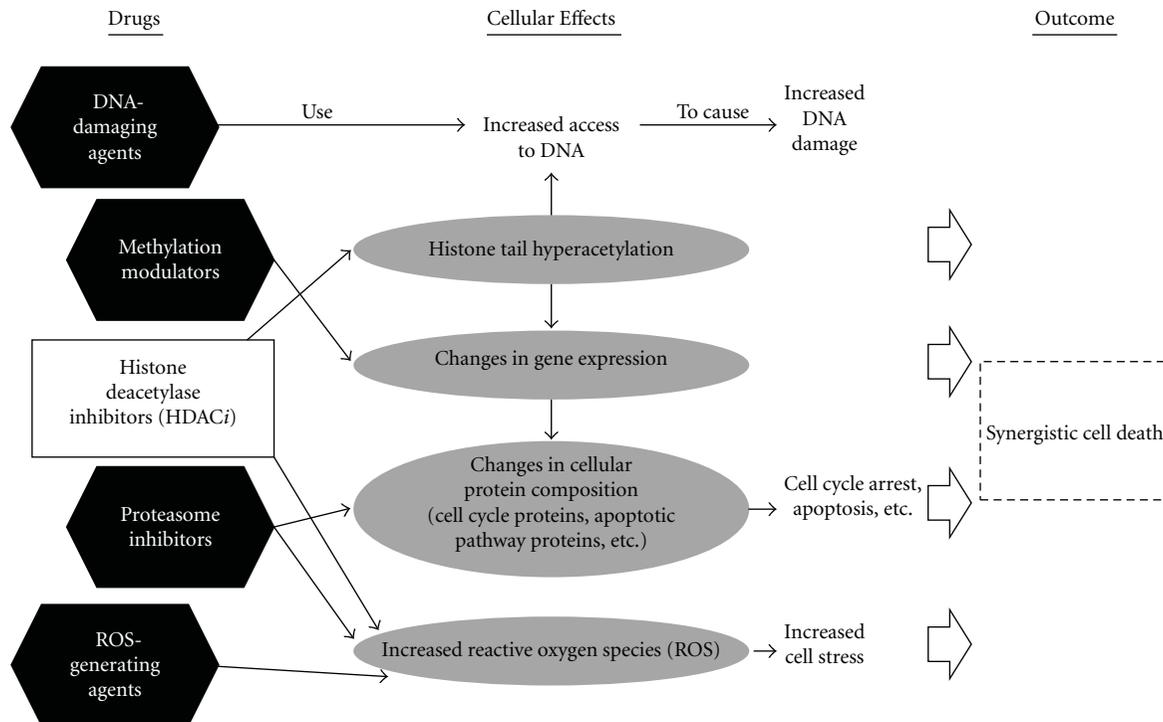


FIGURE 4: Several therapeutic combinations with HDACi show promising results. Some agents which have been shown to cause synergistic cell death when combined with HDACi include (1) DNA-damaging agents, such as radiation and many chemotherapies, which may take advantage of the increased access to DNA, provided by the “loose” chromatin arrangement after HDACi treatment, to cause increased DNA damage, (2) modulators of methylation, such as histone demethylase inhibitors and DNA methyltransferase inhibitors, which have been shown to contribute changes to the methylome that act synergistically with the actions of HDACi, (3) proteasome inhibitors, which increase cellular stress and lead to production of reactive oxygen species, can synergize with HDACi, and (4) ROS-generating agents, such as adaphostin, work with HDACi to amplify ROS production, adding to the already increased ROS levels in cancer cells until toxic levels lead to cell death.

non small cell lung cancer [125] and medulloblastoma [126], FK228 in gastric and colorectal adenocarcinoma [127], PCI-24781 in cervical and colon carcinoma [128], and vorinostat in medulloblastoma [126] and melanoma [129]. There have also been studies in mouse models that indicate that this is an effective combination. Camphausen et al. have found that the treatment of DU145 prostate carcinoma xenografts with entinostat radiosensitized them in a manner that correlated with their ability to cause hyperacetylation in these animals [130]. Clearly, the evidence that HDACi can successfully sensitize cells to radiation is quickly accumulating.

Interestingly, Kim et al. have shown that inhibiting some classes of HDACs seems to be more effective than inhibiting others. This is an important observation since each HDACi has a different inhibition profile. This study showed that the most effective inhibitors were TSA (inhibits class I and II HDACs) followed by SK7041 (inhibitor of class I), while the least effective inhibitor was a suppressor of class III HDACs (splitomicin) [131].

9.2. Topoisomerase II Inhibitors. Many of the same concepts mentioned previously can be applied to combinations of HDACi with various chemotherapies that induce DNA damage. This line of thought led investigators to combine HDACi with topoisomerase II (Topo II) inhibitors. In a study

where several cancer cell lines were treated with either vorinostat or TSA, then subsequently treated with a Topo II inhibitor such as cisplatin or 5-fluorouracil, the combination was shown to be more effective at causing cell death than the chemotherapeutics alone. The investigators in this study also tried to switch the drug order, treating with the chemotherapy first and the HDACi second. This particular administration did not offer any sensitivity advantage over using the chemotherapy as a single agent. The authors proposed that this was evidence that the effectiveness of the combination stems from the ability of HDACi to allow increased access to DNA to the Topo II enzymes, which are then “locked” onto the DNA by the subsequent treatment with Topo II inhibitors, leading to increased DNA damage [132].

9.3. Temozolomide. Temozolomide is an alkylating agent that is commonly combined with radiation for the treatment of gliomas [133]. A study in a glioblastoma cell line showed that the best response may be seen when the HDACi AN-9 was combined with both radiation and temozolomide [134]. Observations of the effectiveness of temozolomide with HDACi have led to several clinical trials, mainly for gliomas (<http://www.clinicaltrials.gov/>). Though trials are mostly still in early stages, preliminary results indicate that the side

effects of combinations such as vorinostat and temozolomide are fairly well tolerated [135]. A few of the combinations of HDACi with temozolomide now in clinical trials can be found in Table 1.

Overall, studies examining the interaction between HDACi and DNA-damaging agents, including radiation or chemotherapeutics, are providing the rationale to combine to these anticancer therapies for clinical utility.

10. Conclusions

Inhibitors of histone deacetylases are promising compounds for the treatment of cancer, and as a result, the first HDACi was approved for CTCL. Many of the cellular outcomes of HDAC inhibition are caused by changes in gene expression that influence growth inhibition, differentiation, and apoptosis of malignant cells. However, due to their limited activity in specific cancer types as single agents, the future of HDACi may reside in combination therapies. Here, we reviewed potential candidates, such as inhibitors of DNA methyltransferases and histone demethylases, ROS-generating compounds, proteasome inhibitors, and DNA-damaging agents, that have demonstrated enhanced efficacy when combined with HDACi and may provide a therapeutic advantage in the clinic (Figure 4). These combination studies offer the rationale to explore these therapies and provide the molecular framework for better therapeutic strategies. Preclinical data suggests that combination treatments may lead to better efficacy and utility of HDACi in the clinic. However, the effects of these combinations on dose-limiting toxicities have not thoroughly been evaluated. Combination studies will offer the opportunity to use lower doses and reduce dose-limiting toxicities, which include fatigue, vomiting, nausea, and diarrhea, among others, that have been observed with HDACi as single agents [39, 45, 50]. While there could be concerns that using combinations of agents may result in increased toxicity, the preliminary data from current clinical trials show promise that combinations can be safe and tolerated. Early data from phase I studies in refractory or relapsed multiple myeloma patients using HDACi/bortezomib regimens indicate similar adverse effects associated with HDACi but no dose-limiting toxicities have yet to be reported [136]. In another ongoing study evaluating vorinostat with 5-azacitidine in a phase I trial in myelodysplastic syndrome ($n = 20$) and acute myeloid leukemias ($n = 8$) patients, preliminary results demonstrate complete response in 43% of participants, while observing grade 1-2 toxicities (fatigue and anorexia) [137]. There are some regimens that are showing response but present toxicities [138]; however, these combinations are currently being tested in phase I trials where the main objective is to try to determine the ideal doses that will improve efficacy with minimal toxicities to patients. Additionally, another important factor to consider is that most participants enrolled in clinical trials have already been exposed to various anticancer agents and chemotherapeutics and already present preexisting conditions that perhaps influence their sensitivity to dose-limiting toxicities. It will not be until

these ongoing trials are complete that we can make a definitive conclusion whether these combination approaches are suitable alternatives for cancer patients. While HDACi are providing anticancer benefits, we cannot exclude that the precise mode of action of how HDACi are killing cancer cells is still unknown. Thus, further understanding of this process may offer insights into how best to use these inhibitors and design better treatment regimens. Much of the work to date focuses on examining gene alterations as a consequence of HDAC inhibition due to acetylation of histones; however, new exciting data seems to suggest that we have to take into consideration other mechanisms. Interestingly, hyperacetylation of nonhistone proteins is linked to the HDACi effect on cancer cells [139]. Transcription factors such as p53, HIF-1, and E2F1, signaling molecule Smad 7, the chaperone protein Hsp 90, and the structural protein tubulin are all substrates of HDACs. A recent study examining the acetylome identified numerous target proteins that can be acetylated. A mass spectrometry study identified over 3,500 lysine acetylation sites in 1,750 proteins [51]. Importantly, they observed an increase in the acetylation of nonhistone proteins in cells treated with HDACi, entinostat and vorinostat. Surprisingly, these HDACi only increase acetylation on 10% of all acetylation sites suggesting that their effects are very selective. As expected, differences in acetylation of substrates were observed between both HDACi. For example, vorinostat was a more potent inducer of histone acetylation and of DNA double-strand breaks, as demonstrated by an increase in γ -H2AX. The nonhistone substrate, Hsp90, was also highly acetylated with vorinostat but not with entinostat. On the other hand, the tumor suppressor p53 was acetylated to a higher degree with entinostat whereas vorinostat did not influence the acetylation of this protein. These differences probably reflect the ability of these compounds to target different HDACs. The identification of the acetylome offers a snapshot of the different proteins that may be influenced by acetylation and therefore by HDACi. These types of experiments that attempt to elucidate the great spectrum of protein acetylation [51] have revealed new targets and unknown potential mechanisms by which HDACi may work as an effective therapy for cancer. Studies like these can be mined to identify molecular end points that can be targeted with more efficacious HDACi-combination therapies.

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Review Article

Autophagic and Apoptotic Effects of HDAC Inhibitors on Cancer Cells

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Because epigenetic alterations are believed to be involved in the repression of tumor suppressor genes and the promotion of tumorigenesis in cancers, novel compounds endowed with histone deacetylase (HDAC) inhibitory activity are an attractive therapeutic approach. Indeed, the potential of HDAC inhibitors for cancer therapy has been explored in preclinical models, and some agents approved for hematologic malignancies have reached the clinical setting. HDAC inhibitors are able to mediate the induction of both apoptosis and autophagy, which are related to anticancer activity in a variety of cancer cell lines. Given the inherent resistance to apoptosis that characterizes cancer, the targeting of alternative pathways is an attractive strategy to improve anti-tumor therapy. The activation of autophagy represents novel cancer treatment targets. This paper aims to critically discuss how the anticancer potential of HDAC inhibitors may elicit a response to human cancers through different cell pathways leading to cell death.

1. Introduction

An ideal anticancer agent should be toxic to malignant cells with minimum toxicity towards normal cells. Currently, there are only a limited number of such agents available for clinical use; thus, the development of novel cancer-selective drugs is an important and challenging task. Caspase-mediated apoptosis, involving both intrinsic and extrinsic pathways, is the main mechanism of action of most current anticancer treatments. Several anticancer drugs have been shown to induce autophagy as well as apoptosis. Autophagy is a catabolic pathway whereby cytoplasmic proteins and organelles are sequestered in vacuoles and delivered to lysosomes for degradation and recycling; however, the role of autophagy in cancer development and in the response to therapy is still controversial, requiring further investigation.

Chemoresistance may be due to epigenetic alterations leading to defects in the apoptotic pathway; therefore, modulating epigenetic changes may increase the efficacy of chemotherapy. It is possible to interfere with epigenetic modifications, such as histone acetylation, using relatively

specific and reversible inhibitors. In addition, the induction of autophagy has been also observed in malignant cells following treatment with histone deacetylase (HDAC) inhibitors [1]. HDAC inhibitors are promising new agents for the treatment of multiple myeloma as they promote hyperacetylation and activate chromatin remodeling. HDAC inhibition leads to many changes at the molecular and cellular levels. HDAC inhibitors preferentially kill transformed cells or cancer cells in both cell cultures and animal models. Although further investigation is required to fully elucidate the consequences of both autophagy and apoptosis induction by treatment with HDAC inhibitors, the pharmacological inhibition of HDAC provides a new therapeutic basis for targeting cell death processes.

2. HDAC Family

In addition to DNA mutations (genetic changes), most cells in multicellular organisms result from aberrations in chromatin-modifying proteins, such as HDAC and DNA methyltransferase (epigenetic changes). It became

increasingly evident that cancer formation and persistence may be caused not only by genetic mutations but also by changes in the patterns of epigenetic modifications [2]. Epigenetic modifications are of profound importance to cells and appear to play roles in many, if not all, biological processes throughout life. In particular, acetylation and deacetylation of the lysine residues on histone proteins play a key role in the regulation of gene transcription [3]. Whereas histone acetyltransferase catalyzes the acetylation of histones and relaxes chromatin to increase the accessibility of transcription factors to the promoters of the target genes, HDACs remove the acetyl groups from histones and repress transcription [4]. In the discovery of drugs, HDAC has become a novel target for the treatment of cancer and other diseases. Inhibition of HDAC activity leads to the acetylation of histones and nonhistone proteins, and alterations in target gene expression are related to changes induced by HDAC inhibition.

According to phylogenetic analyses and sequence homology, the HDAC family consists of at least 18 members divided into two families and four classes in eukaryotic cells. These two families are the classical and sirtuin families. HDACs are categorized into class I (HDACs 1, 2, 3, and 8), class IIa (HDACs 4, 5, 7, and 9), class IIb (HDACs 6 and 10), class III (SIRT), and class IV (HDAC11) [5]. Class I and II have zinc as a cofactor, so they are hydrolases which contain zinc. Class III, which is a series of NAD-dependent Sir2 families, differs from other HDAC classes. Class I and II HDACs are mainly found to be involved in cancer pathogenesis. Class I are found exclusively in the nucleus, whereas in certain cellular signals, class II shuttle between the nucleus and cytoplasm. While human HDACs are well characterized in terms of their catalytic domains, the discovery of type-specific inhibitors is important for anticancer therapies.

3. HDAC Inhibitors

HDAC inhibitors are emerging as potent anticancer agents that can reactivate gene expression and restore the capability of malignant cells to undergo programmed cell death. Several natural and synthetic compounds are currently known to inhibit HDACs (Table 1). Since HDAC inhibitors do not inhibit all HDAC isoforms to the same extent, these agents can be grouped into pan- and class I-specific inhibitors [5]. Hydroxamic acids (TSA, SAHA, LAQ824, and CBHA) and pyroxamic acids (PXD101 and CRA-026440) are pan-HDAC inhibitors targeting class I, II, and IV HDACs in the nanomolar range [17]. In contrast, carboxylic acids (valproic acid and sodium butyrate) and benzamides (MS275, CI-994, and MGCD0103) or cyclic tetrapeptides (trapoxin, desipeptide and spiruchostatin A) are rather class I-specific HDAC inhibitors [18].

HDACs are often overexpressed in various types of cancers, compared with the corresponding normal tissues, and their overexpression is correlated with a poor prognosis [19]. Currently, at least 15 different HDAC inhibitors are being used in clinical trials as a part of mono- or combination therapies. For example, the orally available pan-inhibitor LBH589 (panobinostat) is currently being

developed for several hematological malignancies. SAHA (vorinostat) has been approved for marketing based on data from a large Phase II trial of patients with cutaneous T-cell lymphoma that demonstrated a 30% objective response rate [6]. The class I-selective intravenously delivered agent FK228 (romidepsin), has also shown promising efficacy in patients with cutaneous T-cell lymphoma, with an overall objective response rate of 32%. The class I-selective orally available compound, MGCD0103 (mocetinostat), is in development for multiple solid and hematologic cancers [13]. Clinical trials of several HDAC inhibitors include patients with a wide variety of hematologic and solid neoplasms, including chronic lymphatic leukemia, Hodgkin's lymphoma, myeloproliferative disorder, B-cell lymphoma, acute myeloid leukemia, multiple myeloma, head and neck cancer, brain tumors, melanoma, lung cancer, hepatocellular cancer, breast cancer, ovarian cancer, renal cell carcinoma, and pancreatic adenocarcinoma. However, the main side effects of HDAC inhibitor, SAHA, are fatigue, nausea, anorexia, diarrhea, thrombus formation, thrombocytopenia, neutropenia, anemia, myalgia, hypokalemia and hypophosphatemia. Reversible thrombocytopenia has been seen with almost all HDAC inhibitors in the clinic and appears to be a mechanism-related class effect. By targeting only the most relevant HDAC isoform, it may be possible to greatly improve the efficacy by removing certain toxicities that may be associated with the inhibition of multiple isoforms.

4. Programmed Cell Death (PCD)

Cell death is a result of an unsuccessful cytoprotective mechanism against intracellular and extracellular stressors, and it is broadly divided into three forms: apoptosis, autophagy, and necrosis (Table 2). Morphologically, type I cell death (apoptosis) is characterized by chromatin condensation and DNA fragmentation. Biochemically, apoptosis is characterized by double-stranded cleavage at the linker regions between nucleosomes, resulting in the formation of multiple DNA fragments, and phosphatidylserine externalization accompanied by a series of gene and protein expressions. In the extrinsic pathway, apoptosis is mediated by death receptors on the cell surface, while in the intrinsic pathway, mitochondria play an important role. In both pathways, activated caspases (cysteine aspartic acid-specific proteases) cleave their substrates and activate other downstream cellular substrates. Type II cell death (autophagy) is characterized by the massive accumulation of double-membrane vesicles, commonly referred to as autophagosomes. In general, autophagy promotes the survival of stress; however, there is increasing evidence that when autophagy is overstimulated, it can progress to autophagic cell death. It has been also documented that malignant cell types undergo autophagic cell death when responding to anticancer agents, indicating the potential utility of autophagic cell death induction in cancer therapy. Type III cell death (necrosis) is characterized by oncosis and plasma membrane rupture [20]. Necrosis is an uncontrolled cell death manifesting osmotic dispersion of cells and organelles. While apoptosis involves the rapid destruction of all cellular structures and organelles,

TABLE 1: Characteristics of HDAC inhibitors in clinical trials.

Chemistry	Compounds	HDAC Targets	Clinical trials	Ref
Hydroxymates	SAHA (vorinostat)	Classes I, II, and IV	Phase III*	[6]
	PXD101 (belinostat)	Classes I, and IIa, HDAC6	Phase II	[7]
	Trichostatin A	Classes I and II	Toxic	[8]
	LAQ824 (dacinostat)	Classes I and II	Phase I	[9]
	PCI24781	Classes I and IIb	Phase I	[10]
	LBH589 (panobinostat)	Classes I and IIa	Phase II	[11]
Cyclic tetrapeptides	FK228 (romidepsin)	HDAC1, 2, 4, 6	Phase II	[12]
Benzamides	MGCD0103 (mocetinostat)	HDAC1, 2, 3, 11	Phase II	[13]
	MS275 (entinostat)	HDAC1, 2, 3, 9	Phase II	[14]
Short-chain fatty acids	Valproic acid	Classes I and IIa	Phase II	[15]
	Butyrate	Classes I and IIa	Phase II	[16]

* Approved (cutaneous T-cell lymphoma).

autophagy is a slow, spatially restricted phenomenon in which parts of the cytoplasm are sequestered within double-membraned vacuoles and finally digested by lysosomal hydrolases [21].

5. Apoptosis Induced by HDAC Inhibitors

All HDAC inhibitors have been reported to activate either an extrinsic or intrinsic pathway or both of these cell death pathways in many cancer models [22]. The death-receptor pathway is activated when ligands, such as Fas or TRAIL, bind to their death receptors. It was observed that MS275 (entinostat) as well as SAHA induced TRAIL expression without altering DR4 or DR5 levels in breast cancer cells, which was mediated via SP1 and markedly enhanced adriamycin cytotoxicity in these cells [23]. This resulted in the recruitment of an adaptor protein, FADD, and the activation of caspase-8. The mitochondrial pathway is activated by stress stimuli (chemotherapeutic agents) that disrupt the mitochondrial membrane, causing the release of proteins, including cytochrome *c* and SMAC. Cytochrome *c* release leads to apoptosome formation and activation of caspase-9. Caspase-8 and caspase-9 can then cleave caspases-3, -6, and -7, culminating in apoptosis. SAHA and MS275 induced mitochondrial dysfunction and apoptosis through enhanced ROS generation, XIAP downregulation and JNK1 activation [24]. Various pro- (Bax, Bak, Bim, Bid, etc.) and antiapoptotic (Bcl-2, Bcl-xL, Mcl-1, etc.) proteins regulate these cell death pathways. HDAC inhibitors, including TSA, FK228, SAHA, and LBH589, decrease the expression of Bcl-2, Bcl-xL and XIAP, and enhance the expression of pro-apoptotic proteins, such as Bax and Bak, thereby enhancing TRAIL-mediated cytotoxicity in a variety of cancer cells via the amplification of intrinsic as well as extrinsic apoptotic pathways [25]. Using siRNA approaches targeting Bim, the contribution of Bim to TSA-induced mitochondrial apoptosis was confirmed [26]. In addition, serine protease-dependent and caspase-independent apoptosis is induced by treatment with the pan-HDAC inhibitor, TSA, in pancreatic ductal adenocarcinoma cell lines [27]. This process correlates

with the initially increased expression of the multidomain proapoptotic Bcl-2 family member, Bax, and the subsequent release of AIF and Omi/Htr-A2 from mitochondria. The Bax tumor suppressor gene is downregulated by SIRT1 through the DNA end-binding factor, Ku70. In the absence of SIRT1, Ku70 might be hyper-acetylated, leading to the disruption of Ku70-Bax interaction. This in turn releases more Bax, which promotes apoptosis [28]. HDAC2 inhibition opens the locus of the epigenetically silenced *NOXA* gene, a BH3-only protein and apical initiator of apoptosis [29]. Given the multitude of cellular effects triggered by HDAC inhibitors, it is probable that several different mechanisms contribute to their anticancer activity. Another mechanism of cancer cell death resulting from HDAC inhibitor treatment is discussed below.

6. Autophagy Induced by HDAC Inhibitors

While autophagy has become one of the most attractive topics in cancer research, the current autophagy studies are often viewed as confusing, because of its association with apparently contradictory roles, such as survival and cell death, depending on the model used [30]. A number of anticancer therapies, including HDAC inhibitors, have been observed to induce autophagy in human cancer cell lines.

6.1. Prosurvival Functions. Autophagy is an evolutionarily conserved cell survival pathway that enables cells to recoup ATP and other critical biosynthetic molecules during nutrient deprivation or exposure to hypoxia, which are hallmarks of the tumor microenvironment. The disruption of autophagy with the antimalarial drug, chloroquine, strongly enhanced SAHA-mediated apoptosis in colon cancer cells, knockdown of the essential Atg7 also sensitized cells to SAHA-induced apoptosis [31]. HDAC6, a class II HDAC isotype, is directly involved in the autophagic degradation of the cell; therefore, drugs that impair autophagy could theoretically potentiate the anticancer efficacy of SAHA and other HDAC inhibitors by disabling this important cell survival mechanism.

6.2. Prodeath Functions. The cytotoxic effects of autophagy may be explained by the extensive autophagic degradation of intracellular content or by the interfacing of the autophagic process to proapoptotic signals. Persistent autophagy in response to cellular stress states serves as a potent death signal, as in the case of therapy-induced autophagy, a specific nonapoptotic death pathway triggered upon exposure to chemotherapeutic compounds. In general, autophagic cell death is caspase independent and does not involve classic DNA laddering; however, the mechanisms underlying autophagic cell death are mostly undefined. A link between autophagy and related autophagic cell death has been demonstrated using pharmacological (3-methyladenine) and genetic (silencing of *Atg5*, *Atg7*, and *Beclin-1*) approaches for the suppression of autophagy. In chondrosarcoma cell lines, SAHA induced autophagy-associated cell death, as shown by the detection of autophagosome-specific protein and specific ultrastructural morphology in the cytoplasm [32]. In HeLa cells, HDAC inhibitors (butyrate and SAHA) can induce both mitochondria-mediated apoptosis and caspase-independent autophagic cell death. HeLa cells with APAF-1 knockout or Bcl-xL overexpression were induced to undergo autophagic cell death when cultured with SAHA or butyrate [1]. Therefore, it is reasonable to propose that the induction of autophagic cell death may be used as a therapeutic strategy to treat cancer.

7. Molecular Mechanisms of HDAC-Induced Autophagy

Many signaling pathways, including mTOR, AIF, ROS, CDKs, and HDAC1/6, play important roles in regulating HDAC-induced autophagy (Table 3). Their molecular mechanisms and regulation of autophagy are discussed below.

7.1. mTOR. mTOR plays a role in the initiation and maturation of autophagy by controlling signal transduction cascades involved in this process, acts as a good gatekeeper in autophagy, and exerts an inhibitory effect on autophagy [40]. Both mTOR and phospho-mTOR expressions were strongly decreased in SAHA-treated endometrial stromal sarcoma (ESS-1) cells in a concentration-dependent manner [33]. It seems that SAHA modulates the stability and/or the degradation mechanisms of mTOR at the protein level. SAHA also exerted an influence on mTOR phosphorylation. The level of phospho-S6rp, which plays a regulatory role in the mTOR pathway, was also affected by SAHA. S6rp phosphorylation is directly involved in the control of autophagic processes. The same results were found in ESS-1 treated by rapamycin, a well-known mTOR inhibitor. Further investigations are needed to elucidate the exact pharmacological mechanisms of different HDAC inhibitors in different experimental systems and to clarify whether HDAC inhibitors modulate the expression and phosphorylation of mTOR directly or act somewhere upstream of mTOR.

7.2. AIF. It was examined in malignant rhabdoid tumor cells whether HDAC-inhibitor- (FK228-) mediated autophagy

involved AIF, which acts as a safeguard executioner in cancer cells with faulty caspase activation [34]. FK228 treatment induced the redistribution of AIF to the nucleus, in either the presence or absence of zVAD-fmk. siRNA against AIF prevented the redistribution of LC3 into punctate cytoplasmic structures and blocked FK228-induced autophagy. AIF siRNA also decreased the conversion of LC3-I to LC3-II, as analyzed by Western blotting. Treatment with autophagy inhibitors (chloroquine or 3-methyladenine) increased FK228-induced cell death. These findings suggest that AIF contributes to the autophagy induced by FK228, and disrupting autophagy enhances FK228-induced apoptosis.

7.3. ROS. Targeting the autophagy pathway might enhance the anticancer activity of SAHA, and autophagy inhibitors would synergistically potentiate the proapoptotic effects of SAHA. SAHA has pleiotropic effects that contribute to its mechanism of action in malignant cells, including the generation of ROS, and this has been shown to be a critical event in SAHA-induced cell death [41]. To determine whether chloroquine may modulate SAHA-induced ROS generation, the intracellular levels of the oxygen radical superoxide (O_2^-) were quantified after 12 h treatment with chloroquine, SAHA, and the combination of chloroquine and SAHA in K562 and LAMA 84 cells [35]. In both cell lines, there were marked increases in the generation of O_2^- in cells treated with a combination of chloroquine and SAHA compared with cells treated with either single agent. Cells pretreated with N-acetyl-cysteine displayed significantly reduced apoptosis induction in response to chloroquine and SAHA. It is possible that the initial ROS release triggered autophagy as a protective defense mechanism of cancer cells to deal with SAHA, but the temporal order of protective autophagy is followed by apoptosis, as if autophagy sets the stage for apoptosis to kill prostate cancer cells after a futile attempt to rescue them from death.

7.4. p21^{CIP/WAF1}. Both a novel sulfur-containing hydroxamate HDAC inhibitor, H40, and SAHA induced autophagy in prostate cancer PC-3M cells in a concentration-dependent manner, which correlated well with their cytotoxic effects. These HDAC inhibitors significantly increased CDK inhibitor p21^{CIP/WAF1} mRNA and protein expression in both PC-3M and HL-60 cells in concentrations within their cytotoxic ranges [36]. The upregulation of p21^{CIP/WAF1} correlated with reduced sensitivity and blocked the apoptotic mechanism, and these cells seemed to die by autophagy. Recently, silencing of HDAC3 has been found to induce the expression of p21^{CIP/WAF1} and cell cycle arrest in the G2/M phase in colon cancer cells [42]. HDAC4 was shown to suppress p21^{CIP/WAF1} in ovarian carcinoma cells, cervical cancer cells, glioblastoma cells and breast cancer cells in a nonredundant fashion [43]. These results suggest that the presence of p21^{CIP/WAF1} determines which type of PCD the cell undergoes.

7.5. HDAC1 and HDAC6. HDAC inhibition leads to many changes at the molecular and cellular levels. Class I HDAC

TABLE 2: Characteristic features of programmed cell death.

Programmed cell death (PCD)	Feature	Key regulators	Measurements
Apoptosis (type I PCD)	Chromatin condensation	Caspases	Caspase activation
	DNA laddering	Cytochrome <i>c</i>	DNA fragmentation
	Blebbing (nuclear, cytoplasmic) Apoptotic bodies	Bcl-2 family members	Annexin V
Autophagy (type II PCD)	Blebbing	Autophagy. genes: <i>beclin</i> , <i>LC3</i> , <i>Atg1</i> , <i>Atg5</i> , and <i>Atg7</i>	LC3 localization
	Autophagic vesicles		Autophagic vesicles
	Degradation of Golgi No DNA laddering		
Necrosis (type III PCD)	Swollen organelles	RIPK1, TRAF2, PARP, and Calpains	Extracellular HMGB1
	Random DNA degradation		
	Cytoplasmic membrane rupture Potent inflammatory response		S100 family members

TABLE 3: Effects of HDAC inhibitor on the process of autophagy.

Mechanisms	HDAC inhibitors	Cell lines*	Detection methods	Ref
mTOR	SAHA	ESS-1	Phosphorylation (mTOR)	[33]
AIF	FK228	MRT	siRNA (AIF)	[34]
ROS	SAHA	K562, LAMA84	N-acetyl-cysteine, chloroquine	[35]
p21 ^{CIP/WAF1}	H40, SAHA	PC-3M, HL-60	mRNA, protein expression	[36]
HDAC1	FK228	HeLa	siRNA (HDAC1)	[37]
HDAC6	Tubacin	MEF	siRNA (HDAC6)	[38]
Mitochondria	LBH589, LAQ824	<i>Eμ-myc</i> lymphoma	Knockout (Apaf-1, caspase-9)	[39]

* ESS-1: endometrial stromal sarcoma cells; LAMA84: human chronic myeloid leukemia; MEF: mouse embryonic fibroblasts; MRT: malignant rhabdoid tumors.

inhibitor, FK228, led to autophagic activities through the formation of autophagic vacuoles and the increase of acidic compartments in HeLa cells. When HDAC1 siRNA was used, the amount of HDAC1 mRNA was significantly decreased in knocked-down cells, and HDAC1 inhibition induced the conversion of LC3-I to LC3-II which leads to autophagy in HeLa cells [37]. Thus, HDAC1 may play a role in autophagy because the inhibition of HDAC1 with a specific inhibitor or siRNA can induce autophagy.

HDAC6 is a member of the class II HDAC family and is known to deacetylate α -tubulin and increase cell motility. The ubiquitin-binding deacetylase, HDAC6, was also identified as a central component of basal autophagy that targets protein aggregates and damages mitochondria; however, HDAC6 is not required for autophagy activation; rather, it controls the fusion of autophagosomes to lysosomes [38]. HDAC6 promotes these fusion events by recruiting a cortactin-dependent, actin-remodelling machinery to ubiquitinated protein aggregates, where the assembly of F-actin facilitates autophagosome-lysosome fusion and clearance of autophagic substrates. In addition, the observation that tubacin, a selective inhibitor of HDAC6 deacetylase activity, blocks LC3 recruitment suggests that deacetylation is essential for this process.

7.6. Mitochondrial Function. HDAC inhibitors, LAQ824 (dacinostat) and LBH589, induce the apoptosis of *Eμ-myc* lymphomas via the intrinsic apoptotic pathway. Cells that inhibited the later stages of apoptosis signaling, through the knockout of *apaf-1* or *caspase-9*, displayed morphologic features of autophagy but did not diminish the effects of LAQ824 and LBH589 on mitochondrial membrane permeabilization [39].

8. Relationship between Autophagy and Apoptosis

Recently, several anticancer drugs have been shown to induce not only apoptosis, but also autophagy in cancer cells [44]. The relationship between autophagy and apoptosis is complex and varies with cell types and the specific stress placed upon the cell [45]. While the molecular mechanisms leading to apoptosis have been elucidated to some extent during the past 15 years, autophagic cell death is not well characterized at the molecular level yet [46]. The induction of autophagic cell death may be an ideal approach for cancers that are resistant to apoptosis by anticancer therapies.

It is clear whether autophagy and apoptosis are strictly interconnected, as highlighted by the finding that the two

pathways share key molecular regulators. These are summarized as follows.

- (1) Caspase-3 inhibitor zDEVD-fmk upregulated autophagy in the mouse lung cancer cells [47].
- (2) Following genotoxic stress or oncogene activation, the p53 tumor-suppressor protein is stabilized and activated as a transcription factor, capable of inducing apoptosis. Nuclear localization of p53 induces autophagy, whereas cytoplasmic accumulation inhibits autophagy [48].
- (3) Serum- and amino acid-starved LAMP2-negative cells exhibited an accumulation of autophagic vacuoles and then succumbed to cell death with hallmarks of apoptosis, such as loss of mitochondrial transmembrane potential, caspase activation, and chromatin condensation. Together, the accumulation of autophagic vacuoles can precede apoptotic cell death [49].
- (4) Beclin-1 may be a critical molecular switch that plays an important role in fine-tuning autophagy and apoptosis through caspase-9 [50].
- (5) JNK and p53 may constitute an amplifying loop of autophagic and apoptotic responses. JNK activation results in phosphorylation of Bcl-2, which enhances autophagy by disrupting the interaction between Bcl-2 and Beclin-1 [51].
- (6) The enforced expression of Atg5 not only promotes autophagy but also enhances susceptibility toward apoptotic stimuli irrespective of the cell type, indicating that Atg5 is a molecular switch factor between autophagy and apoptosis [52].
- (7) Full-length Atg5 is an inducer of autophagy, whereas an Atg5 fragment due to cleavage by calpain is proapoptotic [53].
- (8) The formation of autophagosomes occurred early in the sequence of dexamethasone-induced events, before Bak activation, loss of mitochondrial membrane potential, and nuclear fragmentation, showing that dexamethasone-induced autophagy not only lies upstream of apoptosis but is also required for the latter to occur [54].
- (9) A novel Bcl-xL inhibitor, Z36, efficiently induces autophagic cell death in HeLa cells through blocking the interaction between Bcl-xL/Bcl-2 and Beclin-1 [55]. The antiapoptotic protein Bcl-2 is also an anti-autophagic protein through its interaction with Beclin-1 [56].
- (10) The stress induced by N-(4-hydroxyphenyl) retinamide triggers autophagy at a lower dose and apoptosis at a higher dose in glioma cells. At that time, ERK was specifically activated at a lower concentration but not at a higher concentration of retinamide [57].
- (11) The kinetics of Ca^{2+} influx correlated well with the onset of apoptosis and autophagy, suggesting that Ca^{2+} might play a direct role in these processes [58].

BAPTA abolished calcium influx and significantly reduced autophagy and apoptosis.

- (12) Employing 3-methyladenine, a PI3K inhibitor and specific inhibitor of the autophagy pathway, enhanced the apoptotic level [59].

These findings argue against a definitive distinction between type I (apoptotic) and type II (autophagic) cell death. The two pathways are linked in a see-saw manner, such that when apoptosis is inhibited, autophagy is initiated as a backup mechanism and *vice versa* [60, 61].

9. Conclusions

HDAC enzymes affect the acetylation status of histones and other important cellular proteins, which have been recognized as potentially useful therapeutic targets for a broad range of human disorders. In particular, HDAC inhibitors have emerged as major pharmacologic agents for cancer therapy. Most likely, these agents will be used in combination with standard treatment regimens. Efforts to further develop these agents should focus on the thorough evaluation of HDAC expression in different human cancers, the comprehensive analysis of the mechanisms of action of various classes of HDAC inhibitors *in vitro*, and the confirmation of recently identified prognosticators of response in clinical settings. However, most HDAC inhibitors target either all or at least a wide range of HDACs. This creates the problem of the unspecific inhibition of several HDACs, while the targeted blockade of specific single HDACs might be more desirable. Class-specific side effects of pan-HDAC inhibitors have been reported, supporting the requirement of selective inhibitor development. The synthesis of HDAC inhibitors that selectively target HDACs relevant to cancer initiation/progression may enhance the antitumor effects while decreasing the systemic toxicities of HDAC inhibition in cancer patients. It can be expected that the tissue- and time-specific disruption of single HDACs will uncover even more the physiological functions of particular HDACs. Although initial results examining HDAC inhibitors, administered either alone or in combination, have been disappointing, attention has now turned to more potent and selective compounds that may prove to be more effective.

Abbreviations

AIF:	Apoptosis-inducing factor
APAF-1:	Apoptotic protease-activating factor 1
Atg:	Autophagy-related gene
BAPTA:	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
Beclin-1:	Bcl-2-interacting protein
CDKs:	Cyclin-dependent kinases
ERK:	Extracellular signal-regulated kinase
FADD:	Fas-associated death domain
HDAC:	Histone deacetylase
Htr-A2:	High temperature requirement factor A2
JNK1:	c-Jun N-terminal kinase 1
LAMP2:	Lysosome-associated membrane glycoprotein 2

LC3: Microtubule-associated protein 1 light chain 3
 mTOR: Mammalian target of rapamycin
 PCD: Programmed cell death
 PI3K: Phosphatidylinositol 3-kinase
 ROS: Reactive oxygen species
 SAHA: Suberoylanilide hydroxamic acid
 siRNA: Small interfering RNA
 SMAC: Second mitochondria-derived activator of caspase
 S6rp: S6 protein of the 40S ribosomal subunit
 TRAIL: TNF-related apoptosis-inducing ligand
 TSA: Trichostatin-A
 XIAP: X-linked inhibitor of apoptosis protein.

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Review Article

Myelodysplastic Syndrome and Histone Deacetylase Inhibitors: “To Be or Not to Be Acetylated”?

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Myelodysplastic syndrome (MDS) represents a heterogeneous group of diseases with clonal proliferation, bone marrow failure and increasing risk of transformation into an acute myeloid leukaemia. Structured guidelines are developed for selective therapy based on prognostic subgroups, age, and performance status. Although many driving forces of disease phenotype and biology are described, the complete and possibly interacting pathogenetic pathways still remain unclear. Epigenetic investigations of cancer and haematologic diseases like MDS give new insights into the pathogenesis of this complex disease. Modifications of DNA or histones via methylation or acetylation lead to gene silencing and altered physiology relevant for MDS. First clinical trials give evidence that patients with MDS could benefit from epigenetic treatment with, for example, DNA methyl transferase inhibitors (DNMTi) or histone deacetylase inhibitors (HDACi). Nevertheless, many issues of HDACi remain incompletely understood and pose clinical and translational challenges. In this paper, major aspects of MDS, MDS-associated epigenetics and the potential use of HDACi are discussed.

1. Introduction

Myelodysplastic syndromes (MDS) represent a heterogeneous spectrum of haematopoietic disorders ranging from ineffective haematopoiesis with cytopenia to progressive haematopoiesis with transition to acute myeloid leukaemia showing morphological and functional abnormalities of haematopoietic cells [1–3].

Due to difficulties in diagnosis and classification, epidemiological analyses report different incidence rates [4]. Nevertheless, it has been observed that intensive cancer therapeutic regimes lead to higher incidence rates of secondary forms of MDS [5]. As reviewed by Corey et al. [6] and Bernasconi [7], pathogenetic concepts favoured (i) chromosomal

alterations and (ii) gain- and loss-of-function of proto-oncogenes and suppressor genes as well as (iii) disturbance of mitochondrial energy pathway and associated apoptosis. Although good progress was done to develop well-defined step-by-step pathogenetic models such as in colorectal cancer [8–11], the heterogeneous morphological spectrum and different clinical course of MDS remains poorly understood. Therefore, different subgroups of MDS with their characteristic cytogenetic, molecular, and immunological abnormalities were defined by international prognostic scoring systems such as the FAB (French American British) and the WHO classification to help to adequately stratify therapeutic regimens [1, 3, 12]. As described, the primary goal of treatment is haematological improvement in cases with

low-risk MDS and targeting the underlying disease in cases with high-risk MDS [13]. Recently, experimental and clinical investigations revealed that epigenetic processes could play a key role in MDS and could be innovative targets for therapeutic approaches [14–18].

We therefore want to give a comprehensive survey of MDS in the frame of epigenetics with focuses on clinical, pathogenic, and therapeutic issues.

2. A Survey of Myelodysplastic Syndrome (MDS)

2.1. A Short Introduction to the Definition, Classification (with Prognostic Groups), Epidemiology, and Aetiology. According to the WHO, the myelodysplastic syndrome (MDS) is defined as a heterogeneous disease group with cytopenia due to ineffective haematopoiesis and with dysplastic morphological changes in one or more of the myeloid cell lineages and associated risk to progression into acute myeloid leukaemia [1–3].

Based on “characteristic” dysplastic features of haematopoietic cells (in the bone marrow as well as in the peripheral blood) [19–21] five “specific” subgroups of the MDS were distinguished [1, 22], which could be more sophisticatedly subclassified by integrating specific cytogenetic investigations such as MDS with deletion of chromosome 5q done by the WHO in 2008 (as reviewed in detail [2, 3, 22]). Established MDS prognostic groups of low, intermediate I and II as well as of high risk (like the international prognostic scoring system (IPSS)) could identify the individual life risk and could be helpful for therapeutic decisions implementing blast count (according to the WHO classification), the number of cytopenias and cytogenetic findings [12] as well as parameter of red blood cell transfusion [23]. Interestingly, molecular alterations that are linked to specific signalling pathways of MDS like signalling and differentiation, cell cycle regulations, apoptosis, and translation are not integrated into the existing scoring system until now reflecting the morphological and molecular heterogeneity of this haematological entity [13, 22, 23].

MDS could be observed primarily de novo or after radiation or chemotherapy (especially in patients treated with alkylating agents or topoisomerase II inhibitors) as so-called secondary or therapy-associated form of MDS [5, 24–27]. Epidemiological data indicate that especially primary forms of MDS increase with the age of patients [28]: several authors reported an overall incidence rate of MDS ranging between 3.5 to 12.6 per 100,000 population per annum [29–31]. Ageing of the population in the Western world [32–34] and the extensive use of chemo- and radiotherapy for the treatment of malignant tumours [4, 24, 28, 35] will increase the incidence of MDS. Therefore, MDS becomes an important sociomedical issue, as epidemiological investigations revealed an age-specific increase of incidence between the age group of below 70 and above 70 years from 4.9 to 22.8 [36], 1.6 to 15.0 [30], or 15.0 to 49.0 [31], comparable to our own investigations [35].

As discussed above, the linkage between chemotherapy/radiotherapy and therapy-associated MDS is well known.

Yet, knowledge about the aetiology of the large majority of de novo MDS is not fully conclusive, since some of the postulated risk factors for MDS (such as hair dyes, alcohol, and viral disease) showed only a weak or no association with MDS compared to accepted risk factors like solvents, cigarette smoking, and radiation [4, 37]. The inheritance of susceptibility genes is still unclear. Two commonly deleted segments, 5q31 and 7q22, were identified by cytogenetic analysis, which contains tumour-suppressor genes, and are therefore critical regions to MDS development, which could be inherited (germline) or induced by antitumour-therapy (somatic) as reviewed in [38, 39]. Finally, a small percentage of MDS in adults and in children is associated with genetic disorders such as Fanconi anaemia, Bloom syndrome, Diamond-Blackfan syndrome as well as Down syndrome, Shwachman-Diamond syndrome, and neurofibromatosis [6].

2.2. Pathogenetic Insights. As reviewed in detail by Corey et al. [6], Bernasconi [7] and Nimer [18] common and distinct pathways are involved in the pathogenesis of MDS, which could be summarized by (i) chromosomal/genetic alterations and molecular defects, (ii) disturbance of the microenvironment, and (iii) deregulation of apoptosis as discussed in detail below (see also Figure 1).

2.2.1. The Stem Cell Genetic Defect. Cytogenetic investigations revealed a broad range of defects which are linked to specific biological, clinical, and therapeutic features of MDS as reviewed in detail elsewhere [7]. Overall, chromosomal abnormalities could be detected in about 40–60% of primary and in about 70–90% of secondary forms of MDS ranging from balanced/unbalanced chromosomal rearrangements to specific chromosomal abnormalities such as Del(5q), –7, Del(7q), +8, Del(20q), –Y, 17p rearrangements, 11q23 translocations as well as complex karyotypes (≥ 3 defects) [7, 10, 39]. Compared to AML, more deletions and numerical defects than translocations were observed in MDS, which go along with nonclonal defects indicating a different pathogenesis in MDS compared to AML [40, 41]. The role of these chromosomal abnormalities for MDS still remains unclear, since “typical” class I and II mutations in the leukemic transformation of AML are missing in MDS [7, 10, 39], normal and abnormal karyotypes are observed side-by-side in bone marrow of patients with MDS and, finally, chromosomal aberrations are found more often in late than early stages of MDS [42].

Looking on molecular defects in MDS, multiple genes are affected such as CDKN2B, EVI1, IRF1, NRAS, TP53, FLT3, and MLL (in decreasing incidence according to [10]) by mutations, deletions, ectopic expression, or promoter methylation which could influence the expression of tumour suppressor genes, if genetic or epigenetic alterations of the other allele occurred as postulated by Knudson’s hypothesis [43].

Based on the knowledge that the described genetic defect in MDS could be both somatic and/or germline-associated [44–46], the genetic heterogeneity of MDS demand for

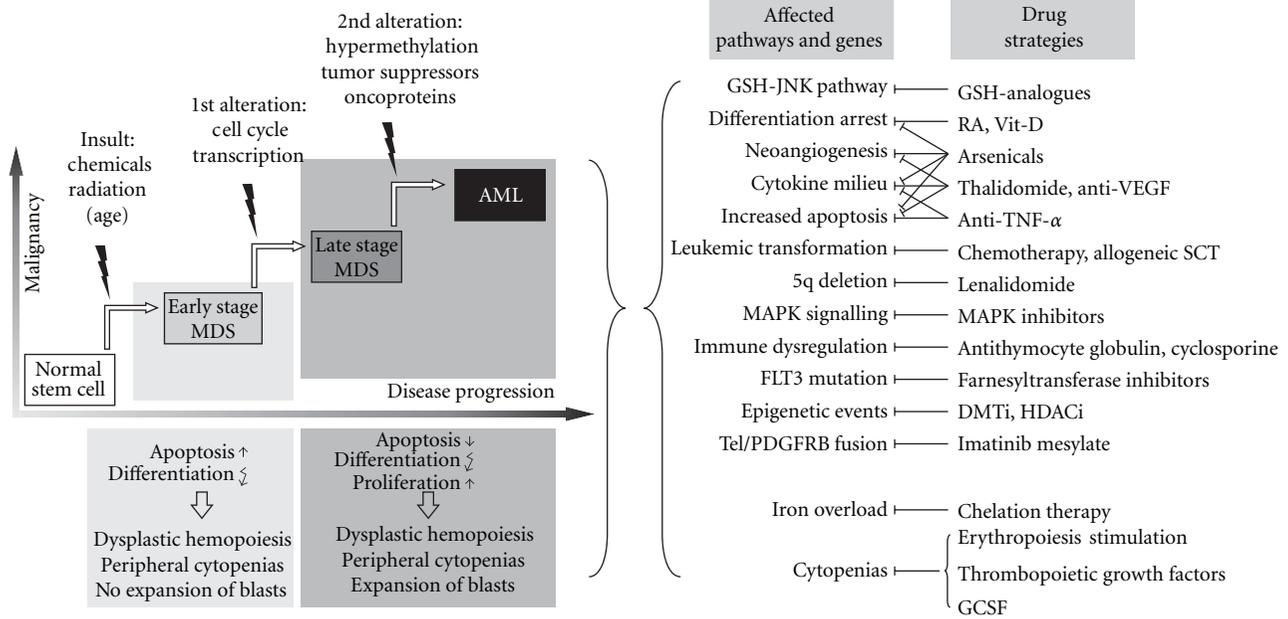


FIGURE 1: Pathophysiological mechanisms involved in MDS and points of action for possible therapy approaches. Abbreviations: AML: acute myeloid leukaemia; G-CSF: granulocyte colony-stimulating factor; GSH: glutathione; MAPK: mitogen-activated protein kinase; MDS: myelodysplastic syndrome; PDGFR: platelet-derived growth factor receptor; RA: retinoic acid; SCT: stem cell transplantation; TNF: tumour necrosis factor; VEGF: vascular-endothelial growth factor.

cytogenetic analysis in each individual case to evaluate the risk of heredity and of disease-progression as well as to develop better therapeutic options [47].

2.2.2. The Role of the Microenvironment. It was shown that abnormalities of the bone marrow microenvironment play a role in the pathogenesis of MDS by abnormal expression levels of cytokines such as interleukin 1 β , interleukin 6, and tumour necrosis factor (TNF)- α [48, 49]. In detail, there is evidence that an enhanced TNF- α expression induced resistance of MDS cells to the proapoptotic effects of TNF- α leading to proliferation and progression [50]. Additionally, deregulation of proangiogenesis factors (like vascular endothelial growth factor (VEGF)) promotes an enhanced self-renewal and cytokine elaboration [51]. In contrast to the findings on VEGF, the expression of matrix metalloproteinases (MMP) (especially MMP2 and MMP9) in monocytes correlated with an increased apoptotic rate and longer overall survival in MDS patients [52].

2.2.3. Apoptosis Deregulation. Deregulation of apoptotic processes is mainly observed in early stages of MDS, whereas a deregulation of proliferation is found in advanced MDS. This is supported by the investigation of apoptosis-associated markers (ligands and receptors) as reviewed by Bernasconi [7]. In low-risk MDS, an upregulation of Apo 2.7, TRAIL, FAS/CD95, p38, TNFR1, CFLARs and Erk1/2 and downregulation of bcl-2, TNFR2, CFLAR_L, NF- κ B, and AKT were found, whereas in high-risk MDS a diametric expression pattern was observed. Apoptosis can be influenced

differentially by cytogenetic defects and by cytokine disarrangement of the stromal cell compartments. For instance, MDS associated with aneuploidies (such as three copies of chromosome 8) presents a significantly higher percentage of apoptotic CD34+ cells. Furthermore, MDS cells with isolated deletion of chromosome 5q showed a G₀/G₁ arrest through the antiproliferative effect of lenalidomide by the adjustment of growth and differentiation signals inside the bone marrow environment [41]. Finally, extrinsic apoptotic pathways could be deregulated by uncontrolled upregulation of apoptosis-associated proteins like FAS/CD95, TNF α , or TRAIL ligands [53].

2.2.4. Molecular Signals for MDS Progression towards AML. What are the essential molecular signals promoting MDS towards AML? Experimental findings investigating apoptotic deregulation suggest a continuous switch from predominant proapoptotic to a more proliferative status of MDS cells [6, 7]. Transcriptional pathways essentially involved in this process are the RELA/NFKB1 and the PI3KC2A/AKT1 signal transduction axis [54, 55]. In short, proinflammatory cytokines (such as TNF α , TRAIL, and FAS/CD95) activate the CHUK/IKBKB complex with consecutive release and nuclear translocation of RELA and NFKB1 along with activation of genes being associated with cell growth, differentiation, inflammation, and apoptosis [7].

In summary, our knowledge of (i) these specific molecular abnormalities in the haematopoietic cells in MDS as well as of (ii) immune deregulation and of (iii) abnormal bone marrow environment in MDS is sophisticated [8–11] (see also Figure 1) and could not explain the heterogeneous

morphological and clinical presentation of this complex disease. Additionally, mouse models of MDS currently available are not suited to reflect all of the features of MDS [56–58]. Taken together, the differentiation as well as proliferation/survival is impaired in MDS with potency to progression to AML due to an unknown second hit event [18].

2.3. Therapeutic Approaches. Standardised therapeutic stratifications were established depending on the prognostic subgroups and with respect to age and performance status of the patients (see also Table 1). The therapeutic aims are: (i) a haematological improvement in low-risk and (ii) altering natural disease course in high-risk MDS disease subgroups. Additional information on clinical and molecular features (as mentioned above) will lead to a tailored, individualised decision management for therapy in future (see Figure 1).

Until now, internationally approved drugs to treat patients with MDS are erythropoietin, darbepoietin, lenograstim (G-CSF), 5-azacytidine, decitabine, anti-thymocyte globulin, cyclosporine, lenalidomide, deferasirox, and deferoxamine [13].

In short, the mechanistic aspects of these currently available treatment options are explained.

- (i) In the “best supportive care” setting erythropoietin, darbepoietin and lenograstim (G-CSF) act as classical hematopoietic growth factors stimulating normal residual hematopoiesis, whereby additional effects of these drugs such as inducing differentiation of dysplastic hematopoiesis via blocking of apoptosis are discussed [61, 62].
- (ii) The chelation therapy with deferasirox and deferoxamine has the intention to reduce the transfusional iron overload associated with organ dysfunction due to chronic anaemia in MDS by mobilization of organ iron deposit and increased secretion of urinary iron [63, 64].
- (iii) Anti-thymocyte globulin, cyclosporine, and lenalidomide have similar immune modulatory properties interacting with deregulated lymphocytes (such as CD4/CD8 ratio or T-cell receptor repertoire) observed in MDS. Additionally, anti-thymocyte globulin and lenalidomide target changes in bone marrow microenvironment in MDS through antiangiogenic and antiproliferative capacities via modifying integrin and chemokine networks. Especially, lenalidomide has the property for direct clonal suppression of myelodysplastic clones with isolated deletion of chromosome 5q [40, 41, 65, 66].
- (iv) The transcriptional modifying therapy contains the two hypomethylating agents 5-azacytidine and decitabine. These two drugs are analogues of the pyrimidine nucleoside cytidine and are integrated into RNA (5-azacytidine) or DNA (both), inducing progressive loss of methylation by covalently binding to DNA methyltransferases which are critical components of the epigenetic network inside normal

and uncontrolled proliferation and differentiation [15, 67].

This heterogeneous list of drugs mirrors the different pharmacological approaches according to the stages and pathomechanisms of MDS. The development of new standardised guidelines for treatment of MDS as done by the National Comprehensive Cancer Network (NCCN) is therefore urgently needed (to view the most recent and complete version of the guidelines, see also <http://www.nccn.org/>) integrating ongoing response findings of clinical trials (e.g. based on epigenetic approaches [14, 16, 18, 68]).

2.3.1. Lower Risk MDS. According to the NCCN practical guidelines for patients with low-risk MDS, a supportive care for symptomatic anaemia and thrombocytopenia is mandatory to additional therapy depending on detectable genetic abnormalities. In cases of del(5q) and other cytogenetic abnormalities, treatment with lenalidomide is indicated. In case of no response and all other remaining cases, the decision of treatment with azacytidine, decitabine, antithymocytes globulin, cyclosporine, or again lenalidomide depends on the serum erythropoietin levels ($<>500$ mU/mL) as described in detail on the NCCN homepage. Additionally, the iron overload should be reduced by the use of iron chelators to reduce the risk of cardiac dysfunction.

2.3.2. Higher Risk MDS. According to the NCCN practical guidelines for patients with high-risk MDS, the intensity of treatment depends on the performance status of the patient and eligibility for allogeneic haematopoietic stem cell transplantation (HSTT). Since the majority of patients with high-risk MDS are relatively old (>70 yr), most of these patients are not possible candidate for high intensity induction chemotherapy and consecutive allogeneic HSTT and therefore receive azacytidine (preferred)/decitabine. The experience with allogeneic HSTT are disillusioning, since the response rate of allogeneic HSTT is generally low in comparison to de novo AML [69, 70]. Newer decision pathways for allogeneic HSTT as well as new induction regimes (such as reduced intensity conditioning) are in development to improve this high-intensity therapy [13].

Additionally, new therapies with heterogeneous pharmacological approaches for MDS are currently developed and investigated in ongoing clinical trials targeting selective pathways within the pathogenesis of MDS showing encouraging results and offering durable benefit to patients with MDS. These new drugs could be sorted according to the targeted mechanism [68, 71, 72]: (i) interaction with survival signals such as antiangiogenesis, receptor tyrosine kinase inhibitors, protein kinase C inhibitors, matrix metalloprotease inhibitors, and farnesyl transferase inhibitors and (ii) interaction with genetic integrity such as immunoconjugate and P-glycoprotein antagonists.

Additionally, an alternative, potential, and promising approach could consist in the application of agents affecting epigenetic pathomechanisms, including histone deacetylase inhibitors (HDACi) such as vorinostat (SAHA), valproic acid, entinostat (MS275/SDX275), or panobinostat

TABLE 1: Therapeutic strategies in MDS depending of risk stratifying (adapted from [59, 60]).

	Low-risk MDS	High-risk MDS
Survival	3–10 years	<1.5 years
Risk of AML transformation	Low rate	High rate
WHO entities	RA, RARS, RCUD, RCMD, MDS-U, MDS del(5q)	RAEB (–1, –2)
IPSS Score (see [12])	Low, Int-1 (score 0-1.0)	Int-2, high (score ≥ 1.5)
	Growth factors: Erythropoietin, G-CSF	Decitabine, 5-azacitidine
	Immune therapy: steroids, cyclosporin, antithymocyte globulin	Investigational
Approved and applied drugs/therapies	Lenalidomide: 5q31 Decitabine, 5-azacitidine Iron chelation	Intensive chemotherapy (Younger, karyotype diploid), allogeneic stem cell transplantation Iron chelation
	Translocation (5;12) or 5q23 variant (PDGFR-B): Imatinib	Translocation (5;12) or 5q23 variant (PDGFR-B): Imatinib
Future therapeutic perspectives	Combination with specific HDAC-Inhibitors	

Abbreviations. AML: acute myeloid leukaemia; G-CSF: granulocyte-colony-stimulating factor; IPSS: International Prognostic Scoring System; MDS-U: MDS unclassifiable; MDS del(5q): MDS associated with isolated deletion of chromosome 5q; PDGFR-B: platelet-derived growth factor receptor B; RA: refractory anemia; RAEB: RA with excess blasts; RARS: RA with ring sideroblasts; RCMD: refractory cytopenia with multilineage dysplasia; RCUD: refractory cytopenia with unilineage dysplasia.

(LBH589). This class of agents (as discussed in detail below) are very interesting in the treatment of MDS, since HDACs reveal pleiotropic effects on cell cycle, differentiation, and apoptosis [73, 74] which are linked and deregulated in MDS.

3. Cancer, Epigenetics, and HDACi

3.1. Cancer and Epigenetic: A Short Overview. Carcinogenesis is characterised by different sequential or parallel genetic/epigenetic hits with a gain- and/or a loss-of-function that leads to “hallmarks of cancer” such as proliferation, apoptosis, tissue remodelling, metastasis, and neoangiogenesis, as described in detail by Hanahan and Weinberg in an outstanding review [75]. In recent years, the importance of epigenetic alterations in carcinogenesis processes is emphasised and led to the development of novel therapeutic approaches.

At a glance, epigenetic mechanisms include DNA methylation of cytosine residues inside CpG islands often found within transcriptional promoter regions in the DNA and various histone modifications leading to altered gene expression [76–78] (see also Figure 2).

3.2. The Role of Histone Modifications. Basic histone proteins H2a, H2b, H3, and H4 build an octamer, called nucleosome packing the DNA by coiling into the nucleus [79]. These histone complexes are posttranslationally modified by different levels of methylation, acetylation, phosphorylation, or ubiquitinylation in order to coordinate the regulation of gene transcription—a process referred to as “histone coding” [80]. These acyl modifications of histone proteins are exerted by two groups of highly conserved enzymes called histone acetyl transferases (HAT) and histone deacetylases (HDAC). HAT transfer acetyl groups to ϵ -amino groups of lysine residues in all four histone proteins leading to

an “open” conformation of chromatin allowing subsequent binding of transcription factors, whereas the typical result of deacetylation by HDACs is condensed chromatin associated with transcriptional repression [81]. Interestingly, only 2 to 10 percent of all genes are regulated by this mechanism as demonstrated using gene arrays emphasizing the role of this “histone code” [74, 82, 83]. However, this data displayed the change in global gene expression, and these studies did not investigate single histone acetylation status or functional analysis of histone deacetylation.

Until now more than 30 different HATs have been described and have been divided into two main classes with different cellular distribution. Whereas A-type HATs are found in the nucleus and have a transcriptional role, B-type HATs are located in the cytoplasm [84, 85]. On the other side, at least 18 different HDACs have been published and are categorised into four major groups based on their sequence homology to their respective yeast HDACs [73, 86]: Class I—HDACs (Rpd3-like) with the zinc-dependent isotypes HDAC 1, 2, 3, and 8 are located in the nucleus and act as transcriptional corepressors. Class II—HDACs (Had1-like) with HDAC 4, 5, 6, 7, 9, and 10 are located in nucleus and cytoplasm and show also a transcriptional corepressor function but also mediate a variety of cytoplasmic nonhistone protein modifications [87]. Class III—HDACs (sirtuins) with SIRT1 to 7 are associated with regulation of cell proliferation and cell cycle control. Additionally, HDAC11 represents a separate class (class IV) of HDACs, since HDAC11 is structurally related to both, class I and II HDACs [88].

This subtype of epigenetic mechanisms of histone modification is centrally involved in the regulation of differentiation, proliferation, and tissue maintenance during embryogenesis [89, 90]. In contrast, deregulated epigenetic action of HDACs is observed in various types of human tumours

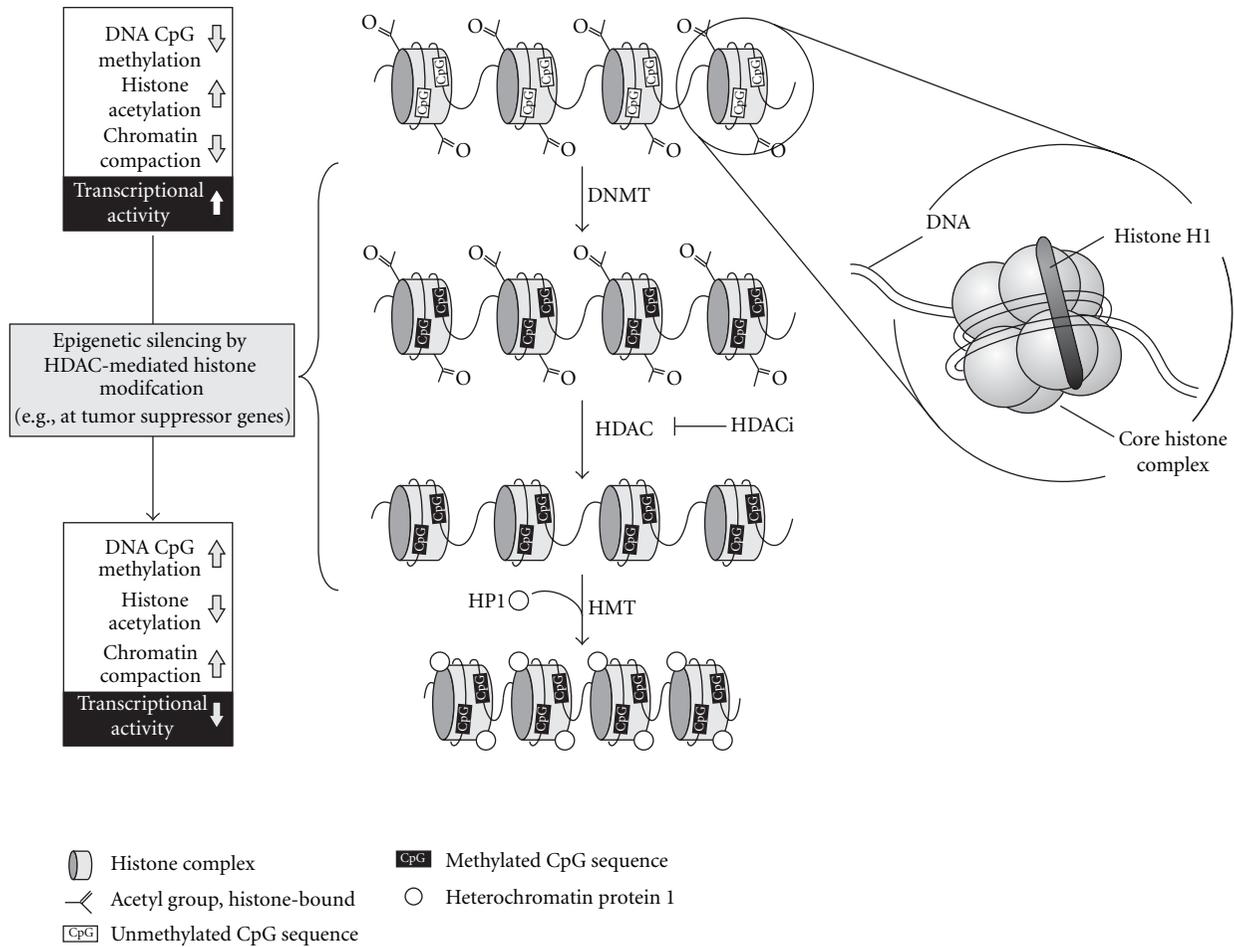


FIGURE 2: Overview of transcriptional regulation by epigenetic mechanisms involving DNA methylation and histone acetylation. Abbreviations: DNMT: DNA methyl transferase; HDAC(i): histone deacetylase (inhibitor); HMT: histone methyltransferase; HP1: heterochromatin protein 1.

such as gastric (HDAC1), breast (HDAC1, HDAC6), or colon carcinoma (HDAC3) [91–94]. Additionally, histone modifications are essentially involved in haematological diseases such as leukaemias as reviewed by Issa [16]. For that reason, the inhibition of HDACs generally seems to be a promising and novel approach in the treatment of human cancer.

3.3. Molecular Classes and Mechanisms of HDACi. Many chemical substances have been developed to inhibit HDACs in vitro and in vivo which could be divided in hydroxamic acid derivatives, cyclic tetrapeptides, benzamides, and short-chain fatty acids as listed in Table 2. Some of them are tested in clinical trials [81, 95], whereas until now only superoylanilide hydroxamic acid (SAHA) has received approval by the FDA for the treatment of cutaneous T-cell lymphoma [96].

In general, the effects of such HDACi are pleiotropic with induction of differentiation, growth arrest, and/or apoptosis of tumour cells [97, 98]. To evaluate the possible role of HDACi in MDS, it is necessary to look into the molecular mechanism of HDAC inhibition. Although the exact mechanisms of anticancer effect by HDACi are under

debate, HDACi have both specific and unspecific effects [74, 81, 99]. With the exception of HDAC6 inhibitors, all HDACi induce a G₁/S-phase cell cycle arrest associated with an increased expression of the endogenous cyclin-dependent kinase inhibitor p21^{cip1/waf1} [100, 101]. This action can be both p53-dependent and -independent as shown by our own experiments [102, 103]. For all other effects observed for HDACi such as upregulation of the death receptor pathway (extrinsic (TRAIL-mediated) and intrinsic (mitochondrial-related)), induction of reactive oxygen species, and alteration of chaperone function (part of the cellular stress response) or NF- κ B pathway (modulator of the inflammatory pathway), it is difficult to define if the effects of HDACi are directly triggered by transcriptional regulation that is mediated by hyperacetylation [87, 104]. Newer investigations revealed that acetylation and deacetylation represent an ubiquitous regulation mechanism for cellular networking such as RNA splicing, DNA damage repair, cell cycle control, nuclear transport, actin remodelling, ribosome, and chaperone function that is summarised as “acetylome” [105–108]. Additionally, HDACi influence posttranscriptional pre-mRNA

TABLE 2: Selected HDAC inhibitors: structural class, compound, isotype selectivity, and study phase—an overview (according to Batty et al. [14] and Schneider-Stock and Ocker [81]).

Structural	HDAC inhibitor (synonyms, abbreviation, supplier)	Class selectivity	Study phase
Hydroxamic acids	m-carboxycinnamic acid bis-hydroxamide (CBHA)		
	Oxamflatin		
	Belinostat (PXD-101, Curagen Corp/TopoTarget A/S)	I, IIa, IIb, IV	II
	Pyroxamide		I
	Scriptaid		
	Superoylamilide hydroxamic acid (SAHA, Vorinostat)	I, IIa, IIb, IV	FDA approval (CTCL)
Cyclic tretapeptides	Trichostatin A (TSA)	I, II	
	Panobinostat (LBH-589; Novartis AG)	I, IIa, IIb, IV	II
	Apicidin	I, II	
	Romidepsin (FK-228, FR-901228; Gloucester Pharmaceuticals Inc)	I, II	II
	Trapoxin-histone acetylase (TPX-HA) analog (CHAP)		
Benzamides	Trapoxin		
	Tacedinaline (CI-994; Pfizer Inc)		
Short-chain fatty acids	Entinostat SNDX-275 (MS-275; Syndax Pharmaceuticals Inc)	I, II	II
	Butyrate	I, IIa	I
	Valproic acid	I, IIa	I

processing and proteasomal and nonproteasomal pathways with heterogenous and different effect on ubiquitination [109], so that it is impossible to define a general mode of action for HDACi [87].

3.4. Clinical Application and Experience of HDACi. The majority of chemically designed HDACi is under intensive clinical investigation for treatment of haematological diseases such as acute or chronic leukaemias, lymphomas, and MDS [110, 111]. Currently, only the pan-deacetylase inhibitor SAHA has been approved by the FDA for treatment of cutaneous T-cell lymphoma (CTCL) [96]. Until now, the overall response reached up to 30%, but long-term surveillance is still missing. From a pharmacokinetic view most of HDACi used in clinical phase I studies have short half-life in plasma (2–8 hrs, except for MS-275 with 80 h [112]), followed by hepatic metabolism and intestinal excretion [113–118]. The major adverse toxicities of HDACi include fatigue, somnolence, confusion, diarrhoea, myelosuppression, and QT prolongation, thus limiting therapeutic applications [113–118]. Additionally, two questions regarding the use of HDACi are still unanswered. (i) It is currently unclear whether more specific HDAC class I & II inhibitors (like MS-275) or pan-deacetylase inhibitors (like SAHA or panobinostat) are more efficient in tumour reduction. (ii) Furthermore, is the acetylation of histone H3 in peripheral blood mononuclear cells a tool for biomarker HDAC inhibitor efficiency [113]? Various studies could not confirm a correlation of peripheral H3 acetylation and tumour treatment responses [110, 111]. Acetylation of peripheral H3 as well as expression of p21 in peripheral blood cells have been considered as potential biomarkers but were shown to possess only a poor correlation with the cognate expression pattern inside a (solid) tumour and with the overall response to the treatment. Additionally, an assay of HDAC enzymatic activity in intact

cells on the basis of a cell-permeate substrate with fluorescent read-out was evaluated in two phase I trials, whereby the reliability of this test is not clear [116, 119]. For that, adequate biomarkers for monitoring tumour target effects of HDACi are still missing.

4. MDS, Epigenetics, and HDACi

It is still under debate how much epigenetics influences initiation and the clinical course of MDS. As mentioned above, experimental data suggest that especially DNA methylation plays an important role in the disrupted haematopoiesis [16]. In the progression of MDS, associated tumour suppressor genes are increasingly methylated, leading to resistance to classical cytotoxic chemotherapy [67]. For instance, methylation frequency of the tumour suppressor genes p15, CDH-1, DAP-Kinases, and SOCS-1 was detected in 89%, 48%, 28%, and 62% of patients with MDS, chronic myelomonocytic leukaemia, and high-risk AML, respectively [120]. Additionally, a genomics-based methylation assay of CD34+ cells of normal control patients and patients with MDS or AML revealed that more than 700 unique genes in CD34+ cells of MDS patients showed hypermethylation compared to normal controls [121, 122]. Recently, mutations of polycomb-associated gene ASXL1 which regulates histone modifications is described in MDS and chronic myelomonocytic leukaemia [123]. Nevertheless, “hard” data on the acetylation status in MDS are missing or are particularly published in circumstance of clinical trials of HDACi or in combination with DNA methyl transferase inhibitors (DNMTi), described below in detail.

4.1. Clinical Trials Phase I/II. In 2001, the HDAC inhibitor valproic acid (VA) in combination with all-trans retinoic acid (ATRA) was shown to induce differentiation in malignant

TABLE 3: Clinical trials of HDACi in MDS and AML (adapted and extended from [15, 17]; see also current and ongoing clinical trials at <http://www.clinicaltrials.gov/>).

Author (year)	HDACi substance	Phase	Schedule	Patient number	Diagnosis: patient number	Responses (i) overall [%] (ii) details	Toxicity
Gore et al. (2001) [127]	Phenylbutyrate	I	i.v., 125–500 mg/kg/day 7/28 days continuous infusion	27	MDS: $n = 11$, AML: $n = 16$	8 [30%] 4 HI, 4 decline of PB blasts	CNS toxicity, hypocalcemia, nausea/vomiting
Gore et al. (2002) [128]	Phenylbutyrate	I	i.v., 375 mg/kg/day 7/14 or 21/28 days cont. infusion	23	MDS: $n = 9$, AML: $n = 14$	2 [9%] 2 HI (21/28 schedule)	CNS toxicity, skin reaction, hypo-calcemia
Zhou et al. (2002) [129]	Phenylbutyrate + ATRA	I	i.v., 200–400 mg/kg/day 25 days	5	AML M3: $n = 5$	1 [20%] 1 RT-PCR neg. CR	Transient CNS depression
Odenike et al. (2006) [130]	Depsipeptide	II	i.v., 18 mg/m ² /dayday 1, 8 and 15 every 28 days	18	AML: $n = 18$	2 [11%] 2 BM- blast clearance (t(8;21) and t(4;21))	Nausea, vomiting, fatigue
Byrd et al. (2005) [131]	Depsipeptide	I	i.v., 13 mg/m ² day 1, 8, 15 every 28 days	10	AML: $n = 10$	Transient declines in PB and BM blasts	Fatigue, vomiting, nausea, tumor lysis syndrome, diarrhea
Giles et al. (2006) [132]	LBH589	I	i.v., 4.8–14 mg/m ² , days 1–7 every 21 days	14	AML: $n = 13$, MDS: $n = 1$	8 [57%] 8 patients transient decline in PB blasts	QT-prolongation, nausea, vomiting, hypokalemia
Garcia-Manero et al. (2005) [133]	Vorinostat (SAHA)	I	Oral, 100–300 mg 2-3 \times /day, 14/21 days	35	AML: $n = 31$, MDS: $n = 3$, CML: $n = 1$	9 [25%] 1CR, 2CRp, 1PR, 5 complete marrow responses	Nausea, vomiting, diarrhea, neutropenia, typhilitis, fatigue
Gojo et al. (2007) [134]	MS-275	I	Oral, 4–10 mg/m ² , 1 \times /week for 2 or 4 weeks	38	AML: $n = 38$	7 [18%] 7 HI, transient decline in PB and BM blasts	CNS toxicity, infections, fatigue, nausea, vomiting

Abbreviations. AML: acute myeloid leukaemia; ATRA: All-trans-Retinoic-Acid; BM: bone marrow CML: chronic myelogenous leukaemia; CNS: central nervous system; CR: complete remission; CRp: complete response with incomplete platelet recovery; HDACi: histone deacetylase inhibitors; HI: haematologic improvement; MDS: myelodysplastic syndrome; PB: peripheral blood; PR: partial response.

myeloid cells [124, 125] inducing the setup of different pilot studies of heterogeneous combination of these two drugs [17]. The response rate within these clinical trials reflects the morphological subtypes of MDS with overall response rates of 8%, 11%, 22%, and 50% in the line with MDS subgroups ranging from IPSS low-risk, intermediate-I, intermediate-II, to high-risk MDS, respectively. Interestingly, most of the responses were observed in the group with low-risk karyotypes [126].

In subsequent years, several clinical trials with other HDACi were started for therapy of MDS (as listed in Table 3). Most of these clinical trials with HDACi are in phase I indicating the preliminary experience with these drugs in MDS. Specific or pan-HDACi were phenylbutyrate (partially in combination all-trans retinoic acid), depsipeptide (romidepsin), LBH589 (panobinostat), SAHA (Vorinostat), and MS275/SDX275 (entinostat) in descending order according to the frequency of use. As these trials were conducted in a Phase I setting, the overall patient number

is low and the primary endpoints were toxicity and safety; response rates, ranging from 9 to 57%, were only secondary endpoints here.

An interesting aspect is the type of “biomarkers” which are used during these trials to describe the effects of HDACi on deacetylation [15, 17]: in the trials with valproic acid in combination with ATRA, the acetylation status of histone H3 and H4 in blood mononuclear cells as well as of the HDAC protein were determined. While missing other possibilities of sufficiently monitoring the bioactivity of HDACi, clinical effects such as transfusion requirements, white blood cell count, or percentage of immature cells (in the peripheral blood or bone marrow) were additionally used.

In summary, single agent clinical trials of HDACi have shown a good safety profile in patients with MDS, although the response rates observed so far are lower than for DNMT inhibitors, which is attributable to the predominant Phase I trial design conducted so far.

4.2. Combination Therapy. Experimental data could demonstrate that DNA methylation interacts with histone deacetylase activity indicating the recursive complexity of epigenetics [135, 136]. Therefore, it was expected that HDACi and DNMTi would show synergistic effects [67, 137]. This synergistic effects could be explained by the known crosstalk between DNA methylation and histone modifications: (i) HDACs are activated by DNMT and by methylcytosine-binding proteins potentiating the gene silencing effect [76, 138], (ii) hypermethylated genes are resistant to re-expression by treatment with HDACis [139], and additionally (iii) DNMTi increases histone methylation and acetylation (such as H3K4) thus activating gene transcription [140]. Nevertheless, more detailed mechanisms of the synergistic effects of HDACi and DNMTi remain to be investigated. Our own experience with the DNMTi Zebularine and SAHA confirmed the synergy on apoptosis, proliferation inhibition, and differentiation in a pancreatic cancer model [141]. Therefore, analysis of sequential application of DNMTi and HDACi were performed *in vitro* and *in vivo* identifying that primary application of demethylating agent (low dose) following by an HDAC inhibitor show the best re-expression levels of hypermethylated genes [137, 142], which is in line with the concept of DNA methylation via the so-called *de novo* DNA methyltransferases during DNA replication [103].

Interestingly, combination therapy of MDS using DNMTi and HDACi is already ongoing (as listed in Table 4) using the combination of decitabine or azacytidine as DNMTi and VA (in one study in combination with ATRA) or phenylbutyrate as HDACi. The overall response rates are optimistic up to 54%, whereas complete response was observed in up to 22%. Nevertheless, the patient numbers of these clinical trials are small due to the Phase I/II setting and are therefore not powered for determining response rates. No unexpected toxicity profiles were seen. Specific details of these studies were in detail: the study of Gore et al. showed that reversed methylation during the first cycle of therapy correlates with therapy response. Interestingly, this was more often accompanied by induction of acetylation of histone H3 and H4 following administration of the DNMTi rather than of the HDACi [143]. Such convincing data could not be obtained using the HDAC inhibitor VA. In the study of Garcia-Manero et al., global methylation and p15 promotor methylation did not differ between responders and nonresponders. Looking at HDACi effects, histone acetylation did not increase until application of highest dose level of VA, whereas the HDACi target p21^{WAF1/CIP1} increased during therapy [144]. Clinical benefit was observed in the trial of Blum et al. independently of whether with or without VA, confirming that this agent is not a potent HDACi [145]. Finally, the data of the study of Soriano et al. corroborated the findings of the other studies with VA that global mutation as well as induction of p21 and p14 mRNA did not correlate with therapy response [146].

In summary, the combination of HDACi with DNMTi as well as other combinations (including different cytotoxics, targeted therapies and radiation therapy as reviewed from Batty et al. [14]) still remains an interesting field for

experimental investigations as well as for larger randomised trials based on available preclinical data in order to detect the best synergy of these agents.

5. Conclusion and Outlook

The heterogeneous nature of MDS demands differential therapy strategies, which reflects on the one hand prognostic subgroups, age, and performance status of the patients with MDS and on the other hand the associated pathogenesis pathways (see Figure 1 and Table 1). Until now, detailed insights into the pathogenesis of MDS have not been published. Yet, the factors driving progression as well their mechanism of interaction are still unclear. New insights came from the field of epigenetics, which admittedly leads to more complexity, too. First clinical trials give evidence that patients with MDS could benefit from epigenetic treatment with DNA methylation inhibitors and HDACi [14]. Nevertheless, many issues of HDACi remains completely unknown and pose clinical and translational challenges [74].

- (i) As HDACi have been approved in the treatment of CTCL by the FDA, the mechanism of their selectivity is speculative postulating preferential induction of apoptosis *in vitro* [148], expression of HDAC2 in aggressive CTCL [149] as well as modulation of gene expression *in vivo* [150]. For that, further detailed molecular investigations of HDACi treated CTCL are urgently needed to better understand the molecular mechanisms of the reported excellent clinical results in CTCL and to confer these findings to other tumorous diseases like MDS.
- (ii) As histone H3 and H4 acetylation are not correlated with clinical response [110, 111], surrogate markers have to be identified for therapy prognosis, controlling and terminating related to the patient and MDS-related disease stage.
- (iii) An additional task is to clarify specific pharmacological aspects of HDACi such as potency, isotype selectivity, application, and toxicity profile as well as mechanisms of resistance. These findings could support the decision on which HDACi are suitable for which MDS subgroups [151–154].
- (iv) Finally, the sequential application strategy of HDACi with DNMTi or other cytotoxic drugs should be determined to optimize the additive or synergistic effects in the treatment of MDS [14].

For that, we are at the beginning of establishing a HDAC-inhibitor strategy in the complexity of therapeutic management of MDS.

Conflict of Interests

Matthias Ocker is a member of the scientific advisory board for panobinostat by Novartis Pharma GmbH. All other authors have no conflict of interest regarding this paper.

TABLE 4: Clinical trials of combination regimen with DNMTi and HDACi (adapted and extended from [15, 17]; see also current and ongoing clinical trials at <http://www.clinicaltrials.gov/>).

Author (year)	Schedule		Patient number	Diagnosis: patient number	Response <i>n</i> [%]				Toxicity
	DNMTi	HDACi			Overall	CR	CRp	PR	
Garcia-Manero et al. (2006) [144]	DAC 15 mg/m ² days 1–10+	VA orally 20, 35, 50 mg/kg (days 1–10)	54	AML: <i>n</i> = 48 MDS: <i>n</i> = 6	12 (22%)	10 (19%)	2 (3%)		CNS toxicity
Soriano et al. (2007) [146]	AZA 75 mg/m ² day 1–7+	VA orally 50, 62, 5 and 75 mg/kg (days 1–7) + ATRA 45 mg/m ² /day (days 3–7)	53	AML: <i>n</i> = 49 MDS: <i>n</i> = 4	22 (41%)	12 (22%)	3 (5%)	7 (13%) BM responses	CNS toxicity
Maslak et al. (2007) [147]	AZA 75 mg/m ² days 1–7+	PB 200 mg/kg for 5 days after AZA	10	AML: <i>n</i> = 8 MDS: <i>n</i> = 2	3 (30%)		3 (30%)		CNS toxicity, fever, nausea, fatigue
Blum et al. (2007) [145]	DAC 20 mg/m ² days 1–10+	VA escalating doses (days 5–21) 15, 20 or 25 mg/kg	11	AML: <i>n</i> = 11	6 (54%)	2 (18%)	2 (18%)	2 (18%) CRi	CNS toxicity, myelosuppression, infection, myeloid differentiation syndrome
Gore et al. (2006) [143]	AZA 50 mg/m ² days 1–14, 1–10 or 1–5; 75 mg/m ² days 1–5; 25 mg/m ² day 1–14+	PB 375 mg/kg/day for 7 days after AZA	32	AML: <i>n</i> = 18 MDS: <i>n</i> = 13 CMML: <i>n</i> = 1	11 (38%)	4 (14%)	1 (3%)	6 (21%) HI	CNS toxicity, mild nausea, injection sidereactions, asthenia, myelosuppression

Abbreviations. AML: acute myeloid leukaemia; ATRA: all-trans-Retinoic-Acid; AZA: azacytidine; CMML: chronic myelomonocytic leukaemia; CNS: central nervous system; CR: complete remission; CRi: complete responses with incomplete blood count recovery; CRp: complete response with incomplete platelet recovery; DAC: decitabine; DNMTi: DNA methyl transferase inhibitors; HDACi: histone deacetylase inhibitors; HI: haematologic improvement; MDS: myelodysplastic syndrome; PB: phenylbutyrate; VA: valproic acid.

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Review Article

Modulation of Acetylation: Creating a Pro-survival and Anti-Inflammatory Phenotype in Lethal Hemorrhagic and Septic Shock

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Histone deacetylases (HDACs) play a key role in homeostasis of protein acetylation in histone and nonhistone proteins and in regulating fundamental cellular activities. In this paper we review and discuss intriguing recent developments in the use of histone deacetylase inhibitors (HDACIs) to combat some critical conditions in an animal model of hemorrhagic and septic shock. HDACIs have neuroprotective, cardioprotective, renal-protective, and anti-inflammatory properties; survival improvements have been significantly shown in these models. We discuss the targets and mechanisms underlying these effects of HDACIs and comment on the potential new clinical applications for these agents in the future. This paper highlights the emerging roles of HDACIs as acetylation modulators in models of hemorrhagic and septic shock and explains some contradictions encountered in previous studies.

1. Introduction

Hemorrhagic shock (HS) is a major cause of morbidity and mortality among trauma patients. Sepsis or septic shock is a leading cause of mortality in intensive care units. Even if some patients with HS survive the acute episode of blood loss, they often exhibit a systemic inflammatory response syndrome (SIRS), which is often further complicated by the subsequent development of septic shock resulting from a harmful or damaging host response to infection [1]. HS-induced systemic response shares many features with septic response [2]. At the molecular level, it has been reported that both hemorrhage and sepsis lead to an imbalance in acetylation of proteins and that HDACIs can induce protein acetylation and restore this balance [3–5].

1.1. Lysine Acetylation and Histone Deacetylase Inhibitors. Lysine acetylation or N^ε-acetylation, identified initially on core histones in 1968 [6], is mediated by a group of enzymes called histone acetyltransferases (HATs) that transfer acetyl

groups from acetyl-coenzyme A to the ε-amino group of lysines. HATs are counterbalanced by the activity of histone deacetylases (HDACs) that catalyze the hydrolytic removal of acetyl group of lysines. In humans, HDACs are divided into four classes (Table 1) based on their homology to yeast HDACs [7, 8]. Class I HDACs include HDAC1, 2, 3, and 8; these are related to the yeast enzyme Rpd3. Class II HDACs include HDAC4, 5, 6, 7, 9, and 10, which are related to the yeast protein HDA1 (histone deacetylase-A1); class II HDACs are further divided into two subclasses—IIa (HDAC4, 5, 7, and 9) and IIb (HDAC6 and 10)—according to their structural similarities. Class III HDACs are referred to as sirtuins owing to their homology to the yeast HDAC Sir2. This class includes SIRT1–SIRT7 [9, 10]. HDAC11, the most recently identified isoform, is a class IV HDAC due to its distinct structure [11]. Class I, II, and IV are zinc-dependent enzymes whereas class III HDACs are nicotinamide adenine dinucleotide (NAD⁺)-dependent enzymes. Based on their various subcellular localization, intratissue variation, and nonredundant activity, the different HDACs are implicated

in various specific cellular processes, such as proliferation, metabolism, and differentiation. For example, class I HDACs are mainly nuclear enzymes whereas class II HDACs localize either to the cell nucleus or to the cytoplasm, depending on their phosphorylation and subsequent binding of 14-3-3 proteins. Moreover, class I HDACs are ubiquitously expressed [12–15] whereas class II HDACs display a tissue-specific expression.

To date, more than 15 HDACIs have been tested in preclinical and early clinical studies for cancer therapy [16]. Many of them are broad-spectrum- or pan-HDACIs which inhibit many of the Class I, II, and IV isoforms, including suberoylanilide hydroxamic acid (SAHA), trichostatin A (TSA), and valproic acid (VPA). Some clinical compounds such as MS-275, FK-228, and apicidin have been termed as “Class I-selective”, since they target several Class I isoforms of HDAC. Tubacin is one of a few HDACIs that have been reported as HDAC6-specific inhibitor [18]. Suramin, a class III HDAC inhibitor for SIRT1, SIRT2, and SIRT5, is a polysulfonated polyaromatic symmetrical urea with antiproliferative and antiviral activity [17] (Table 1). So far, almost all of the studies focus on class I and class II HDACs [19], and a few focus on class III/sirtuins.

1.2. Protein Acetylation Balance. Signals that enter the cell nucleus encounter chromatin where gene expression takes place. Regulation of gene expression has two components that act in concert: alteration of chromatin structure governed by histone modification and binding by transcription factors (TFs) including activators and repressors. Most importantly, two classes of enzymes control the alteration and binding: HAT and HDAC. HAT modify core histone tails by posttranslational acetylation of specific lysine residues and create appropriate “histone code” for chromatin modification to enhance DNA accessibility of TFs. In general, acetylation of core histones unpacks the condensed chromatin and renders the target DNA accessible to transcriptional machinery, hence contributing to gene expression. In most cases, the TFs can also be acetylated by HAT to facilitate their interactions with DNA and other proteins for transactivation. By contrast, deacetylation of the histones and TFs by HDAC increases chromatin condensation and precludes binding between DNA and TFs, leading to transcriptional silencing. In normal conditions, protein concentration and enzyme activity of HAT and HDAC remain in a highly harmonized state of balance, which is named as “acetylation homeostasis” to emphasize the importance of regulated acetylation in acceding cellular homeostasis [20]. Recent studies have shown that during various neurodegenerative challenges, the balance is disturbed by loss of HAT activity while the ratio of HAT to HDAC tilts in favor of HDAC. The impaired acetylation homeostasis can cause transcriptional dysfunction and facilitate neurodegenerative cascade, which has been implicated in pathogenesis of several neurodegenerative disorders [21, 22]. Indeed, perturbation of acetylation homeostasis is emerging as a central event in the pathogenesis of neurodegeneration. Recent studies have demonstrated HDACIs to be protective in animal models of Huntington’s disease [23–26], amyotrophic lateral sclerosis

[27, 28], experimental autoimmune encephalitis [29], spinal muscular dystrophy [30, 31], and others [32].

Recently, HDACIs have emerged as potent pro-survival and anti-inflammatory drugs, offering new lines of therapeutic intervention for hemorrhagic shock and septic shock. We and other groups have found that HDACIs such as VPA, SAHA, and TSA prevent hemorrhage-associated lethality in a rat and swine models of hemorrhagic shock [3, 4, 33], suppress expression of proinflammatory cytokines, and improve survival in a mouse model of septic shock [5, 34, 35]. We have also demonstrated that inhibition of HDAC can modulate the immune response following trauma/hemorrhage and inflammatory second hit in animals and humans [36]. This paper highlights the emerging roles of HDACIs as acetylation modulators in models of hemorrhagic shock and septic shock, and explains some contradictions encountered in previously published results.

2. HDACIs in Hemorrhagic Shock

HS represents a global ischemic stress, resulting from acute blood loss. Current treatment for HS focuses on pathophysiology at the level of organ systems: to maintain sufficient tissue perfusion and vital organ function through administration of fluids and blood products and to surgically control the source of hemorrhage. Unfortunately, this resource intensive protocol remains difficult to administer, particularly in austere environments, where advanced surgical interventions may not be immediately available [37]. Moreover, this approach fails to address much of the damage that takes place at the cellular level as a result of hypoperfusion (during hemorrhage) and reperfusion (during resuscitation) [38].

Focusing on the cellular pathophysiology of hemorrhagic shock, our laboratory has explored the strategy of pharmacologic resuscitation with HDACIs as the protective agents. HDACIs alter the acetylation status of proteins and therefore have the potential to modulate the genomic and proteomic changes induced by hemorrhage. We have shown that HDACIs can dramatically improve survival in lethal models of hemorrhagic shock in rat [39, 40] and swine models [41]. Moreover, these inhibitors can protect cells from apoptosis and suppress expression of proinflammatory cytokines [5, 42, and Fukudome et al., unpublished data].

The cell protective mechanisms involve (1) epigenetic regulation through post translation modification of histone proteins, (2) activation of cell survival factors such as the PI3-kinase/Akt signaling pathway, (3) blockage of gut-liver/lymph-lung axis, and (4) inhibition of inflammatory mediators such as the Toll-like Receptor 4 (TLR4) signaling pathway. All of these actions directly or indirectly involve restoration of protein acetylation.

2.1. Acetylation-Related Epigenetic Regulation. Control of epigenetic regulation with induction of histone acetylation is the strategy to restore and maintain the normal ratio of HAT/HDAC for treatment of many diseases. A number of preclinical studies have demonstrated that HDACI can improve survival in degenerative diseases, prevent brain from various insults, attenuate the effects of aging, and increase

TABLE 1: Classification of HDACs and selected HDACIs.

HDAC Class	HDAC isoforms	Localization of HDAC	Specific HDAC inhibitors	Nonspecific HDAC inhibitors	References
Class I (Zn ⁺⁺ -dependent)	HDAC1	Nucleus	MS-275, FK-228	TSA	[9, 16]
	HDAC2	Nucleus	FK-228, apicidin	SAHA	
	HDAC3	Nucleus	MS-275, Apicidin	Butyrate	
	HDAC8	Nucleus		Valproic acid	
Class IIa (Zn ⁺⁺ -dependent)	HDAC4	Nuc/Cyt		TSA	[9, 16]
	HDAC5	Nuc/Cyt		SAHA	
	HDAC7	Nuc/Cyt		Butyrate	
	HDAC9	Nuc/Cyt		Valproic acid	
Class IIb (Zn ⁺⁺ -dependent)	HDAC6	Mainly Cyt	Tubacin	TSA	[16]
	HDAC10	Mainly Cyt		SAHA	
Class III (NAD ⁺ -dependent)	SIRT1	Nuc/Cyt	Suramin		[9, 17]
	SIRT2	Nuc/Cyt	Suramin, AGK2		
	SIRT3	Nuc/Mitoch*			
	SIRT4	Mitochondria		Nicotinamide	
	SIRT5	Mitochondria	Suramin		
	SIRT6	Nucleus			
	SIRT7	Nucleus			
Class IV (Zn ⁺⁺ -dependent)	HDAC11	Nuc/Cyt		TSA SAHA	[16]

life span [23, 43–45]. Our group has reported that administration of HDACIs protects organs and cells from lethal hemorrhagic shock-induced injury [36, 42, 46, 47]. Several converging lines of inquiry suggest that the protective mechanism of HDACIs may result from the fact that alter the response to ischemic injury and reduce damage in the important organs during the progress of hemorrhagic shock.

In the heart, ischemia induces histone deacetylase activity via deacetylation of histones H3/4 *in vitro* and *in vivo* [48]. Using standard murine model of heart ischemia-reperfusion, Granger demonstrated that HDACI significantly reduce infarct area, even when delivered 1 h after the ischemic insult. HDACI decrease the response to ischemic injury and lessen the size of myocardial infarction [48]. In part, this is through prevention of ischemia-induced activation of gene programs that include hypoxia inducible factor-1 α , cell death, and by decreasing vascular permeability *in vivo* and *in vitro*, which reduces vascular leak and myocardial injury.

In the liver, low oxygen increases HDAC1, 4, and 5 protein levels by 2-fold and decreases acetylated histone H3 levels to 50%–75% of the control values in a turtle model of anoxia [49]. In a rat model of hemorrhagic shock, Gonzales reported that hemorrhage increased serum levels of lactate, lactate dehydrogenase, aspartate aminotransferase, and alanine aminotransferase. Alternatively, treatment with VPA (an HDACI) induced acetylation of histones (H2A, H3, and H4), and alleviated serum levels of these enzymes and prolonged survival (fivefold). Furthermore, hyperacetylation of the histone proteins indicated the presence of active genes and correlated with improved survival [50]. Gene

expression profiling data from our group, in comparison to HS without treatment, has shown that VPA treatment upregulates expression of 17 genes at the early stage of HS, compared to HS without treatment (Fukudome et al., unpublished data). Two of these genes are peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α) and dual specificity protein phosphatase 5 (DUSP5). PGC-1 α protects cells from oxidative stress by increasing expression of various antioxidant defense enzymes including superoxide dismutase and glutathione peroxidase [51]. DUSP5 is an inducible, nuclear, dual-specificity phosphatase, which specifically interacts with and inactivates the extracellular signal-regulated kinase (ERK) 1/2 MAP kinases in mammalian cells [52]. Inactivation of ERK1/2 MAP kinases by DUSP5 may be one of the mechanisms responsible for the protective properties of VPA in HS.

In the kidney, it has been discovered that ischemia/reperfusion induces a transient decrease in histone acetylation in proximal tubular cells. This is likely a result of a decrease in histone acetyltransferase activity as suggested by experiments with energy-depleted renal epithelial cells in culture [53]. During recovery after transient energy depletion in epithelial cells, the HDAC isozyme HDAC5 is selectively downregulated in parallel with the return of acetylated histone. Knockdown of HDAC5 by RNAi significantly increased histone acetylation and bone morphogenetic protein-7 (BMP7) expression [53]. In a rat model of HS, it was found that treatment of animal with VPA or SAHA markedly increases phosphorylation of Akt and decreases the expression of proapoptotic BAD (Bcl-x1/Bcl-2 associated death promoter)

protein in kidney tissue [54]. Further investigation is needed to find if there is any relationship between HDAC5 inhibition and Akt activation.

In the brain, Faraco found that in the ischemic brain (subjected to 6 h of middle cerebral artery occlusion), histone H3 acetylation levels are dramatically decreased without evidence of a concomitant change in histone acetyltransferase or deacetylase activities. Treatment with SAHA (50 mg/kg i.p.) increased histone H3 acetylation within the normal brain (of approximately 8-fold after 6 h) and prevented histone deacetylation in the ischemic brain. These effects were accompanied by increased expression of the neuroprotective proteins heat-shock protein 70 (Hsp70) and B-cell lymphoma 2 (Bcl-2) in both control and ischemic brain tissue 24 h after the insult. At the same time point, mice injected with SAHA at 25 and 50 mg/kg had smaller infarct volumes compared with vehicle-receiving animals (28.5% and 29.8% reduction, $P < .05$ versus vehicle). Recently, Li reported that VPA treatment induces acetylation of histone H3, increases expression of β -catenin and Bcl-2 proteins, and prevents neuronal apoptosis in *in vitro* hypoxic condition (0.5% O₂) as well as in the *in vivo* model of HS [42]. These findings demonstrate that pharmacological inhibition of HDAC promotes expression of neuroprotective proteins within the ischemic brain and underscores the therapeutic potential of molecules inhibiting HDAC for HS-induced brain injury.

2.2. Effect of HDACIs on Phosphoinositide 3-Kinase (PI3K)-Akt/PKB Pathway. Activation of PI3K enhances cell survival and antagonizes apoptosis via Akt/PKB activity in many cell types including cardiomyocytes, cardiac fibroblast, vascular smooth muscle cells (VSMCs), endothelial cells and hepatocytes [41, 55, 56]. Alam recently found that VPA exerts antiapoptotic effect through the Akt/PKB signaling pathway and improves survival in a swine model of traumatic hemorrhagic shock [41]. In this *in vivo* study, they compared cell protective effects of the HDAC inhibitor (VPA) with resuscitation using fresh whole blood (FWB) and a normal saline control. VPA treatment increased the levels of activated Akt, deactivated glycogen synthase kinase-3 β (GSK-3 β), β -catenin, and Bcl-2 significantly when compared to FWB and saline control groups. However, the total Akt and total GSK-3 β levels did not differ across the three treatments [41]. VPA has been reported to directly and indirectly inhibit GSK-3 β [57]. However, it is not clear how the HDACI influences the Akt signaling in an animal model of trauma and hemorrhagic shock. There are several other possibilities for this pathway activation in addition to direct inhibition of GSK-3 β (Figure 1).

2.2.1. An Increase of Acetylated Tribbles (TRB) 3 and Phosphatase and Tensin Homolog (PTEN) May Be Associated with PI3K/Akt Activity [58]. Growth factors such as insulin, insulin-like growth factor 1 (IGF-1), erythropoietin, and cytokines that reduce apoptosis rely almost exclusively on the PI3K/Akt pathway whereas GPCR-induced PI3K/Akt activation and cardioprotection occurs in response to several peptide agonists, including urocortin, ghrelin, and

adrenomedullin as well as beta2-adrenergic receptor (β_2 -AR) stimulation [55, 59–61]. In the heart, overexpression of Akt/PKB enhances apoptosis in response to myocardial ischemia [62]. Consistent with a critical role for Akt/PKB in cell survival, loss or gain of TRB3 and PTEN activity leads to reduced or enhanced apoptosis, respectively [63, 64]. Alternatively, increased expression of TRB3 and PTEN promotes apoptosis in cardiac myocytes [63, 65]. PTEN is a dual protein/lipid phosphatase whose main substrate is phosphatidylinositol 3,4,5-triphosphate (PIP3), the product of PI3K. PTEN degrades PIP3 to an inactive form phosphatidylinositol 4,5-bisphosphate (PIP2) [55, 66–68], inhibiting Akt activation. PTEN is constitutively active and is the major downregulator of PI3K/Akt [69]. PTEN also forms signaling complexes with PDZ domain-containing adaptors, such as the MAGUK (membrane-associated guanylate kinase) proteins. These interactions appear to be necessary for the metabolism of localized pools of PIP3 involved in regulating actin cytoskeleton dynamics. Acetylation is a major mechanism that regulates PTEN activity [70]. Histone acetylase p300/CREB-binding protein-associated factor (PCAF) interacts with PTEN and acetylates lysines 125 and 128 which are located within the catalytic cleft of PTEN and are essential for PIP3 specificity. PCAF functions as a negative regulator of PTEN [71]. TRB3 is an intracellular pseudokinase that modulates the activity of several signal transduction cascades. TRB3 has been reported to inhibit the activity of Akt protein kinases [72]. TRB3 gene expression is highly regulated in many cell types, and hypoxia or endoplasmic reticulum (ER) stress promotes TRB3 expression. TRB3 binds to inactive and unphosphorylated Akt, thus preventing its phosphorylation [73]. It remains unknown whether and how PCAF regulates TRB3. Recently, Yao and Nyomba reported that acetylation status of TRB3 and PTEN is decreased in association with increased HDAC and decreased HAT activities. The hypoacetylated TRB3 and PTEN can inhibit the Akt-activity in a rat model of prenatal alcohol exposure [58], which suggests that HDACI treatment could inhibit the activity of TRB3 and PTEN, and therefore enhance Akt signaling.

2.2.2. Induction of Hsp70 by HDACIs May Be Associated with the PI3K/Akt Pathway. In a rat model of hemorrhagic shock, Gonzales found that VPA treatment increased the acetylation of nonhistone and histone proteins and expression of Hsp70 in rat myocardium, and significantly prolonged survival (5 fold) compared to the nontreatment control [4]. In rat cortical neurons, VPA treatment markedly upregulated Hsp70 protein levels, and this was accompanied by increased Hsp70 mRNA levels and promoter hyperacetylation and activity [74]. Other HDAC inhibitors—sodium butyrate, trichostatin A, and Class I HDAC-specific inhibitors MS-275 and apicidin—all possess the ability to induce HSP70.

Hsp70 is a molecular chaperone, cell-protective, and anti-inflammatory agent. Marinova recently reported that HDACIs increase Sp1 acetylation, and promote the association of Sp1 with the histone acetyltransferases p300 and recruitment of p300 to the Hsp70 promoter. Furthermore,

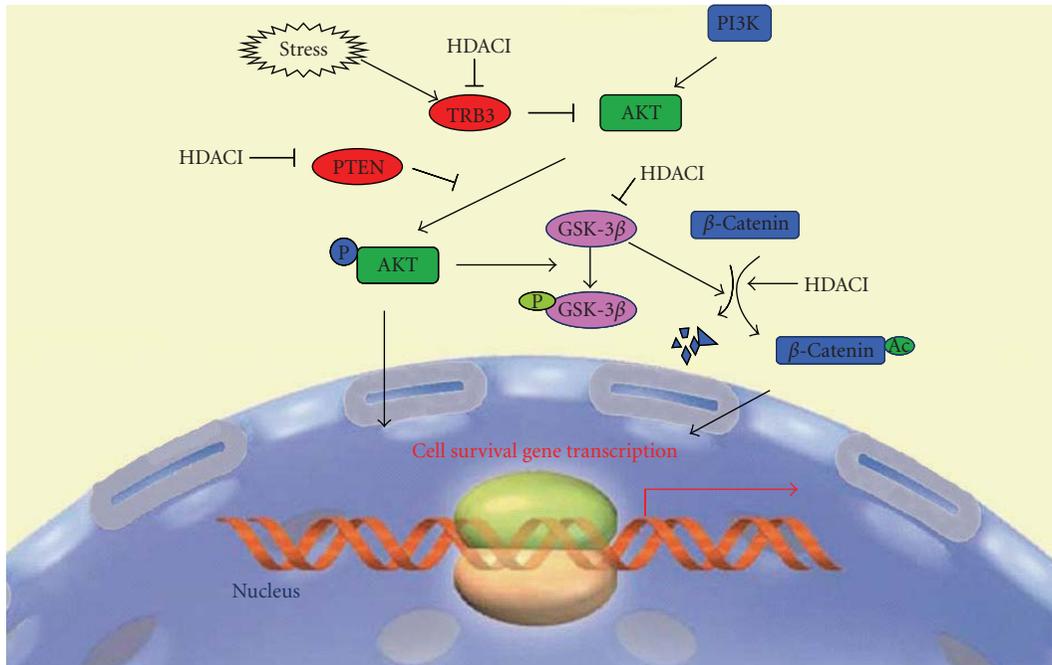


FIGURE 1: Effect of HDACs on cell survival in hemorrhagic shock. HDACs induce phosphorylation of AKT by inhibition of TRB3 and PTEN. While AKT stimulates transcription of cell survival genes through several other pathways, phosphorylated AKT also phosphorylates GSK-3 β . Phosphorylated GSK-3 β becomes inactivated form, which cannot degrade β -catenin. HDACs (e.g., VPA) can also directly inhibit GSK-3 β . Moreover, HDACs induce acetylation and nuclear translocation of β -catenin, leading to downstream survival gene transcription. P, phosphorylation; Ac, acetylation.

HDACI-induced cell protection can be prevented by blocking Hsp70 induction [74]. In addition, Gao and Newton showed that Hsp70 directly binds and stabilizes Akt/PKB as well as protein kinase A and protein kinase C, thus prolonging the signaling lifetime of the kinases [75]. Taken together, these findings suggest that the PI3K/Akt pathway and Sp1 are likely involved in Hsp70 induction by HDACs, and this induction may in turn interact with Akt/PKB to sustain the active state of Akt to protect the cells from apoptosis.

In addition to the interaction with the Akt/PKB pathway, Hsp70 also directly interacts with different proteins of the tightly regulated programmed cell death machinery, thereby blocking the apoptotic process at distinct key points. For example, Hsp70 can inhibit the apoptotic cascade [76, 77], decrease formation of the functional apoptosome complex [78, 79], prevent late cascade-dependent events such as activation of cytosolic phospholipase A2 and changes in nuclear morphology, and protect cells from forced expression of caspase-3 [80]. Moreover, Hsp70 inhibits c-Jun N-terminal kinase (JNK)-mediated cell death by suppressing JNK phosphorylation either directly and/or through the upstream SEK kinase [81–83], and hampers TNF-mediated apoptosis by inhibition of ASK-1 [84].

It has been noted that Hsp70 has different functions depending on its intracellular or extracellular location. Intracellular Hsp70 has a protective function which allows the cells to survive lethal conditions [85]. Extracellularly

located or membrane-bound Hsp70 mediates immunological functions. They can elicit an immune response and will be discussed later.

2.3. Effect of HDACs on Gut and Lung in Hemorrhagic Shock. Hemorrhagic shock is characterized by insufficient tissue perfusion which is needed to meet the oxygen and nutrient demands of cells. The host response to hemorrhagic shock involves a coordinated expression of mediators that act both locally and systemically with profound effects on organ function. The accumulated evidences suggest that gut and lung, especially gut, represent important site(s) of immune mediator production and inflammation. Although two major hypotheses, gut-lymph-lung axis [86, 87] and gut-liver-lung axis [88, 89], have brought much debate based on the different findings, it is clear that hemorrhagic shock is associated with intestinal ischemia which makes gut a proinflammatory organ. For the fact that most of these mediators are produced by cells of the immune system, significant immunoregulatory actions occur within the gut-liver and/or lymph-lung axis and in peripheral immune sites (Figure 2). It is well established that the gut plays a pivotal pathogenic role in the pathogenesis of SIRS and multiorgan dysfunction syndrome (MODS) [90, 91].

It is clear that gut ischemia can cause lung injury, but how can it be prevented? We proposed that HDACs could protect lung from gut-originated damage. To test this

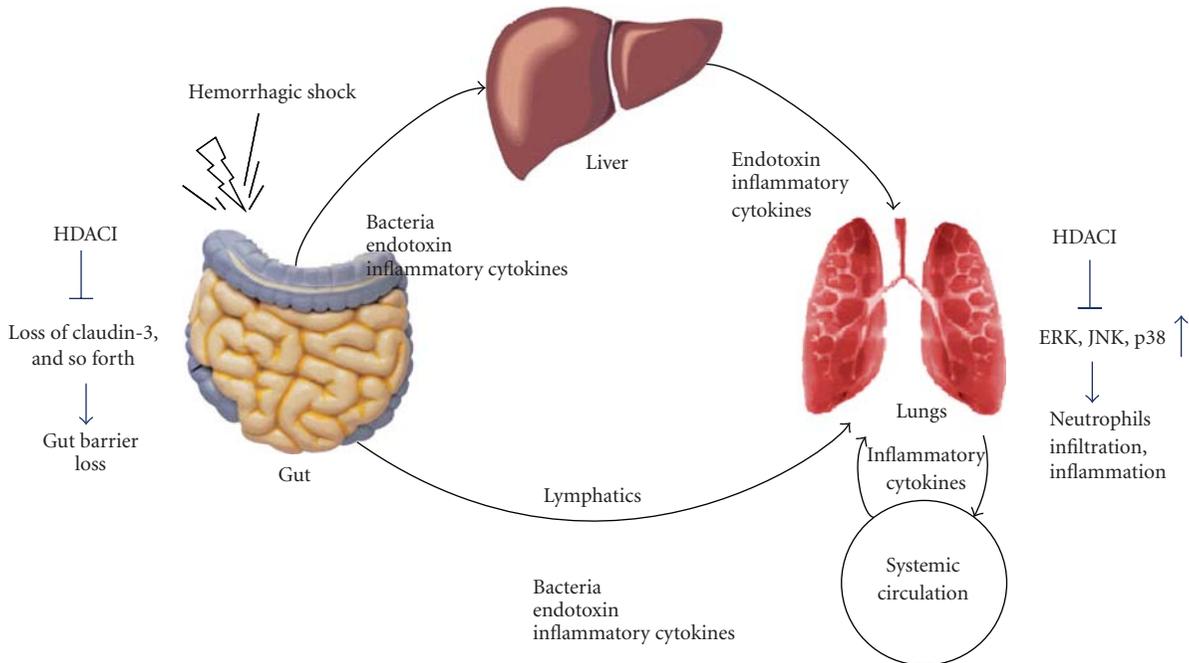


FIGURE 2: Effect of HDACIs on gut-liver/lymph-lung axis in response to hemorrhagic shock. Hemorrhagic shock causes destruction of the gut barrier due to tight junction protein (e.g., claudin-3) loss. Bacteria, endotoxin, and inflammatory cytokines enter into circulation and lung. In the lung tissue, MAPKs are stimulated and neutrophils infiltrated, resulting in acute lung injury. HDACIs block these processing by inhibition of tight junction protein loss in gut and inactivation of MAPKs in lung.

proposal, the superior mesenteric artery (SMA) of rats was clamped for 60 min to induce ischemia and then released for reperfusion. Without any treatment, gut ischemia induced production of proinflammatory cytokines or prostaglandin-like compound such as IL-6, cytokine-induced neutrophil chemoattractant (CINC), 8-isoprostane in lung tissues, and increased neutrophil lung infiltration. However, treatment with VPA significantly reduced these mediators in lung tissues and improved survival in a rat model of ischemia and reperfusion (Kim, unpublished data). It is not clear how VPA protects gut from ischemic damage and prevents acute lung injury. However, based on our recent findings, it is conceivable that VPA maintains gut barrier in part through stabilizing intestinal tight junctions (TJ) (Figure 2).

The function of gut barrier is based on intestinal tight junctions. Encircling epithelial cells, the intestinal TJ is a region where the plasma membrane of epithelial cells forms a series of contacts that appear to completely occlude the extracellular space and create an intercellular barrier and intramembrane diffusion fence [92]. Normally, TJ is anchored in the cell via TJ proteins and filamentous actin (F-actin) cytoskeleton. Hypoperfusion, or ischemia, can cause disruption of F-actin cytoskeleton with subsequent TJ loss and barrier failure. Bacterial translocation to mesenteric lymph nodes, liver, and spleen is found at a very early stage of hemorrhagic shock [89]. Loss of gut wall integrity not only leads to paracellular leakage of microbial products [93, 94] but also contributes to the development of systemic inflammation and distant organ failure [95].

One of the major TJ proteins is claudin-3. Although the exact function of claudin-3 within TJ is not completely clear, it appears to be important in TJ formation and function [96]. Recent studies from our group [97] and others [89] have shown that HS leads to destruction of the gut barrier due to TJ protein loss. Also, it was found that the claudin-3 protein is released into circulation very early (30–60 min) after the onset of HS [97]. Alternatively, CINC, a chemokine that promotes neutrophil chemotaxis, is significantly elevated in serum and lung tissue with increased myeloperoxidase (MPO) levels at 4 h after hemorrhage. However, VPA treatment reverses HS-induced claudin-3 loss from the intestine and reduces the levels of CINC and MPO in serum and lung significantly (Fukudome, unpublished data). These findings suggest that VPA can stabilize claudin-3 in gut TJ, maintain the intestinal barrier, and prevent harmful gut-derived substances from getting into the systemic circulation.

Furthermore, we recently demonstrated that sublethal hemorrhagic shock (40% blood loss) results in phosphorylation (activation) of ERK, JNK and p38 mitogen-activated protein kinase (MAPK) in lung tissues at 1 and 4 hours compared to the sham group. Postshock administration of VPA (300 mg/kg, iv) significantly attenuates the MAPK activation but does not affect expression of total ERK, JNK and p38 proteins [98]. These kinases are globally expressed and known to be key regulators of stress-mediated cell fate decision. Activation of these proteins has been strongly associated with poor outcomes while inhibition of MAP kinases has been associated with survival in hemorrhage models [98].

VPA can also directly modulate MAPK activation. Cao has studied the effects of HDACI treatment on LPS-induced activation of p38 MAPK and found that HDACIs inhibit p38 phosphorylation. In this experiments, HDACIs induce acetylation of MAP kinase phosphatase-1 (MKP-1), a protein that dephosphorylates MAPK and inactivates MAPK pathways. These results demonstrated that HDACI treatment and MKP-1 acetylation increases the interaction between MKP-1 and p38 MAPK, and results in p38 inactivation, reduced inflammation and increased survival among LPS-exposed mice [34]. Whether similar acetylation-mediated mechanisms exist for the regulation of ERK and JNK is still unknown but is highly plausible.

3. HDACIs in Septic Shock

The progression of infection to septic shock begins with the release of inflammatory mediators at the local site of microbe invasion. This induces the migration of white blood cells and platelets to the infection site and contributes to endothelial damage and increased microvascular permeability. Blood flow is also reduced which sets the stage for ischemia-reperfusion injury. These physiologic processes are part of the exaggerated SIRS, which can lead to MODS.

Many of the body's reactions to infection with gram-negative organisms are due to lipopolysaccharide (LPS), a component of the outer bacterial cell wall membrane, also referred to as endotoxin. It can induce septic shock physiology and has been used extensively to produce shock in laboratory models. LPS exerts the downstream signals through the Toll-like Receptor-4 (TLR4). TLR4 activates two downstream pathways: Myeloid differentiation factor 88 (MyD88)-dependent and MyD88-independent pathways. The former leads to the production of proinflammatory cytokines such as IL-6, TNF- α , and IL-12 with the quick activation of nuclear factor-kappaB (NF- κ B) and MAPK. The MyD88-independent pathway is associated with activation of interferon (IFN) regulatory factor 3 (IRF3), subsequent induction of IFN- β , and maturation of dendritic cells.

It has been shown that HDACIs exert anti-inflammatory activities via the suppression of inflammatory cytokines and nitric oxide [99]. In LPS-stimulated human peripheral blood mononuclear cells, HDACI (ITF2357) reduces the release of TNF- α , IL-1 β , and IFN- γ [100]. Other HDACIs such as TSA and SAHA have been shown to decrease LPS-induced inflammation in mice [5, 34, 101]. In RAW 264.7 cells, treatment of the macrophages with SAHA significantly suppresses LPS-induced gene expression and protein production of IL-1 β , IL-6, and TNF- α [5, 101]. In an *in vivo* rodent model of septic shock, HDACIs attenuate acute lung and liver injury and improve survival [35, 101, 102]. Further mechanistic studies have demonstrated that HDACIs play an important inhibitory role in TLR-4-MyD88 signaling pathways via NF- κ B and MAPKs. Protein acetylation provides a key for the control of inflammatory response (Figure 3).

3.1. HDACIs Affect NF- κ B Activity. NF- κ B is an ubiquitously expressed transcription factor that plays an important role

in innate immunity and other critical processes. The NF- κ B family consists of p50, p52, p65 (Rel A), c-Rel, and Rel B, which form homo- or hetero-dimers. The p50/p65 heterodimer is the most frequently found combination in mammals. Inactive NF- κ B complexes are retained in the cytoplasm by the I κ B inhibitor. In innate immune signaling, host cells can respond to the threat of bacterial pathogens (e.g., LPS) via extracellular receptor TLRs (e.g., TLR4). TLR4 interacts with MyD88 and recruits interleukin-1 receptor-associated kinase 1 (IRAK1) and IRAK4 to the receptor complex. IRAK1 phosphorylates TNF receptor-associated factor 6 (TRAF6) leading to the activation of the I κ B kinase (IKK). The activation of IKK results in I κ B phosphorylation, triggering its ubiquitination and proteasomal degradation. Free NF- κ B then translocates to the nucleus to regulate the transcription of chemokines, cytokines, and other inflammatory response molecules [103].

In the nucleus, p50 and p65 can be regulated by acetylation. The function of acetylated NF- κ B is complicated. Acetylation of p50 at K431, K440, and K441 promotes higher DNA-binding affinity towards NF- κ B target sequences correlating with increased p300 (histone acetyltransferase) recruitment and transcriptional activation [104, 105]. The p300 can acetylate p65 at multiple lysine residues and result in different consequence. Acetylation of p65 at K221 and K310 is associated with an increased transcription of NF- κ B target genes [106] and is required for the full activity of p65 [107]. In contrast, HDAC1 and HDAC3 deacetylate p65 at either K221 or K310, resulting in the inhibition of NF- κ B. Additionally, K122 and K123 acetylation reduces p65 DNA-binding affinity accompanied with increased I κ B interaction and nuclear export [108]. The p300-mediated acetylation of K314 and K315 in p65 has no obvious effect on NF- κ B DNA binding or translocation.

Indeed, HDACIs have been shown to induce hyperacetylation and repress NF- κ B signaling and expression of several target genes [109–111]. Conversely, other groups reported that HDACIs enhance NF- κ B-dependent gene expression but were in the presence of TNF α [112–115]. Presumably, inhibitory or enhancive effects of HDACIs on NF- κ B rely on the cell type, expression of a different set of HDAC isoforms, acetylation/deacetylation of NF- κ B at different lysine or by different enzyme, as well as the source of cell stimulation (e.g., LPS, cytokines and high glucose levels) [99].

3.2. HDACIs Inhibit MAPK Activity. In mammalian cells, JNK and p38 MAPKs activate mitogen and stress-activated protein kinase 1 (MSK1) such as ribosomal S6 kinase 2 (RSK2). RSK2 has a strong activity towards phosphorylation of histone H3 at Ser10 [116]. The phosphorylation of histone H3 occurs on the promoters of the subset on the stimulus-induced cytokine and chemokine genes, recruits NF- κ B to the promoters, and stimulates transcription of inflammatory genes such as IL-6, IL-8, IL-12, and macrophage chemoattractant protein 1 (MCP-1) [117].

It has been reported that HDAC inhibitor TSA enhances the activity of mitogen-activated protein kinase phosphatase-1 (MKP-1) [34, 118]. MKP-1 is a nuclear-localized dual-specificity phosphatase and preferentially dephosphorylates

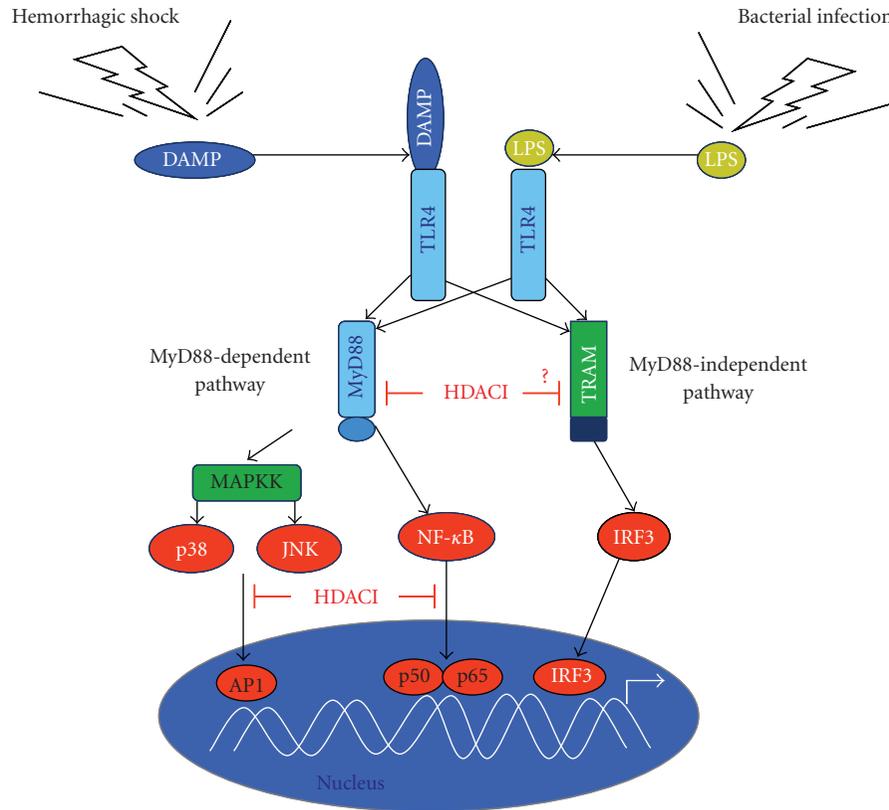


FIGURE 3: TLR4 signaling – a converged immune response pathway for hemorrhagic shock and septic shock. TLR4 not only serves as a key sensor of pathogen-associated molecular patterns (PAMPs) but also is proposed recently to act as a receptor for some endogenous molecules called “alarmins”. HDACIs block TLR4 signaling at multiple steps; therefore, they can inhibit immune response for both hemorrhagic shock and septic shock.

MAPKs such as p38 and JNK. Recently, Cao showed that MKP-1 interacts with HAT and that acetylation of MKP-1 inhibits TLR4 signaling [34]. They immunoprecipitated the histone acetylase p300 and showed that it was associated with MKP-1. Moreover, MKP-1 was acetylated by p300 on lysine residue K57 within its substrate-binding domain. Acetylation of MKP-1 induced by TSA enhanced the interaction between MKP-1 and p38 MAPK, suggesting that HDACI could increase the phosphatase activity and inactivate p38 MAPK. Indeed, TSA increased MKP-1 acetylation and blocked MAPK signaling in wild-type (WT) cells. However, TSA had no effect in cells lacking MKP-1. Furthermore, TSA reduced inflammation and mortality in WT mice treated with LPS, but failed to protect MKP-1 knockout mice. These findings suggest that acetylation of MKP-1 inhibits innate immune signaling, and targeting the MAPK pathway by HDACI may be an important approach in the treatment of septic shock.

Recently, our group has found that HDAC inhibitor SAHA can reduce the expression of MyD88 gene and protein *in vitro* and *in vivo* after LPS insult [101]. Moreover, SAHA acetylates heat shock protein 90 (Hsp90) and deassociates the protein from IRAK1, resulting in IRAK1 degradation (Chong, unpublished data). Our new findings have provided evidence that inhibition of HDAC can block, at least in part,

activity of NF- κ B and MAPKs in the initial steps of the TLR4-MyD88- NF- κ B/MAPK pathway (Figure 3).

3.3. TLR4 Signaling – a Converged Immune Response Pathway for Hemorrhage and Sepsis. Hemorrhage and sepsis activate several inflammatory and innate immune signaling pathways [119]. Systemically, these pathways promote recruitment of neutrophils and release of inflammatory cytokines [120]. Within the cells, the inflammatory stimuli induce MAPK-dependent phosphorylation or phosphoacetylation of histone proteins and modulate the epigenetic accessibility of DNA in the cell [117]. Downstream, these signals change the gene expression profile of the cells insulted with hemorrhage or sepsis, altering the competing signals (e.g. prosurvival and prodeath, or anti-inflammation and proinflammation), that ultimately determine their fate. Two key pathways in hemorrhage- and sepsis-induced cellular injuries are the mitogen activated protein (MAP) kinase and NF- κ B pathways. These proteins, ERK1/2, JNK, p38 protein kinase, and NF- κ B, are globally expressed and known to be key regulators of cell fate decisions [121, 122], which are involved in TLR4 signaling pathway.

It is well known that mammalian TLR4 serves as a key sensor of pathogen-associated molecular patterns (PAMPs)

such as LPS. More recently, an additional role for TLR4 has been proposed. A number of reports have emerged to suggest that diverse molecules of host-cell origin may also serve as endogenous ligands of TLR4 [2, 123, 124]. These molecules represent members of a recently identified family of molecules, including Hsp70, fibrinogen, high-mobility group box 1 (HMGB1), nucleolin, and annexins [123]. They have been found to serve as mediators of inflammation that may be expressed or released in response to tissue damage from trauma including HS. These molecules have been described as “alarmins”, which are the equivalent of PAMPs but are endogenous molecules. They are rapidly released following nonprogrammed cell death but are not delivered by apoptotic cells. Immune cells can also be induced to produce and release alarmins without dying. Generally, this is done by using specialized secretion systems or by the endoplasmic reticulum (ER)-Golgi secretion pathway. Endogenous alarmins and exogenous PAMPs can be considered subgroups of a larger family of damage-associated molecular patterns (DAMPs). They convey a similar message and elicit similar responses through TLR4 (Figure 3) leading to activation of MAPKs and NF- κ B pathways [123, 125].

HDACs have been described above for their pro-survival and anti-inflammatory properties. The combined pro-survival and anti-inflammatory effectiveness makes them a highly attractive choice for the treatment of lethal hemorrhagic shock and its septic complications. In our preliminary studies, we have already discovered that HDACs not only inhibit expression of proinflammatory cytokines and chemokines in cells but also prevent some alarmins from being released from cells in hemorrhagic shock and septic shock (Li, unpublished data). Further investigation with different models (e.g., “two-hit” model) are being planned to further clarify the precise mechanisms of action and the role played by protein acetylation.

4. Contradiction and Explanation

Although most studies have demonstrated that HDACs induce expression of pro-survival/anti-inflammatory genes and proteins, some authors have reported different results [126]. Wetzal et al. found that TSA causes inhibition of glioma cell growth by both cell cycle arrest and apoptosis [127], which contradicts the study of Avila et al. that TSA increases neurons survival [31]. Aung et al. observed that treatment of bone marrow-derived macrophages with TSA enhances LPS-induced expression of proinflammatory genes [128], which disagrees to the finding of Han and Lee that TSA significantly decreases mRNA and protein levels of the proinflammatory cytokines in macrophages [129]. This discrepancy of pro/anti-apoptosis and pro/anti-inflammation may be due to differences in cell types, HDAC isoform, acetylation/deacetylation of a protein by different enzymes or at different lysine residues, and variances in the type of cellular stimulation, and so forth [99].

For instance, HDACs have been shown to potentiate TNF- α expression in microglial cells [130] but to suppress TNF- α expression in cultured human peripheral blood mononuclear cells and macrophages in response to LPS [5, 131].

It is not clear how HDACs function differently in these different cell types. However, there are numerous reasons for the differential effects of HDACs between normal and transformed cells. HDACs induce different phenotypes in various transformed cells, including growth arrest, activation of the extrinsic and/or intrinsic apoptotic pathways, autophagic cell death, reactive oxygen species (ROS)-induced cell death, mitotic cell death, and senescence. In comparison, normal cells are relatively more resistant to HDAC-induced cell death [132, 133]. In many transformed cells, ROS-oxidation-reduction pathways are important mechanisms of HDAC-induced transformed cell death [134]. Thioredoxin (Trx) acts as a hydrogen donor required for the activation of many proteins, including ribonucleotide reductase which is essential for DNA synthesis and transcription factors and is an antioxidation scavenger of ROS [135]. HDACs upregulate the expression of Trx-binding protein 2 (TBP2) [136], which binds and inhibits Trx activity, and can cause downregulation of Trx in transformed but not normal cells [134, 136]. Trx is an inhibitor of apoptosis signal regulating kinase 1 (ASK1) [137]. Therefore, inhibition of Trx by HDACs in transformed cells subsequently results in cell apoptosis.

Differential cellular expression of HDACs is another factor that causes the discrepancy. The HDACs have been found to interact with different proteins and transcription factors. HDAC1 interacts with retinoblastoma protein [138] and SP1 [139]. HDAC2 interacts with NF- κ B [140]. HDAC1 and HDAC2 also interact directly with DNA topoisomerase II to modify topoisomerase activity [141]. HDAC3 interacts with NF- κ B [142]. HDAC4 and HDAC5 interact with myocyte enhancer factor 2 (MEF2) [143]. Due to the protein-protein interaction, inhibition of HDAC with different HDAC inhibitor results in different outcome. In cultured neonatal mouse cardiomyocytes, Zhu et al. have shown that LPS insult increases HDAC3 activity and suppresses TNF- α expression. The upregulation of HDAC3 activity was abrogated by a pan-HDAC inhibitor TSA and class I HDAC inhibitor apicidin, but not by class II HDAC inhibitors [142].

Posttranslational modifications of a protein by different enzymes or at different amino acid residues can cause different results. For instance, phosphorylation of NF- κ B p65 preferentially interacts with p300/CBP, an acetyltransferase, resulting in p65 acetylation at multiple sites. Acetylation of K221 and K310 is associated with an increased transcription of NF- κ B target genes [106], while K122 and K123 acetylation reduces p65 DNA-binding affinity accompanied with increased I κ B interaction and nuclear export [108]. Deacetylation of p65 K310 by SIRT1 inhibits transcription of NF- κ B target genes [144], and deacetylation of p65 at either K221 or K310 by HDAC1 and HDAC3 results in the inhibition of NF- κ B [145].

In addition, a study from Wilson and colleagues suggests that the opposite effects of HDACs may result from the effects of acetylation on specific combinatorial interactions required for efficient transcription of individual genes [146]. They used the mouse mammary tumor virus (MMTV) promoter to assess the consequences of inhibiting histone deacetylase activity on transcriptional activation mediated by the progesterone receptor *in vivo*. In human breast cancer

cells, TSA induced global histone hyperacetylation, and this effect occurred independently of the presence of the hormone. Interestingly, chromatin immunoprecipitation analysis revealed no significant change in the level of acetylated histones associated with MMTV promoter following TSA treatment. In these cells, the MMTV promoter adopted a constitutively “open” chromatin structure. Treatment with TSA converted the MMTV promoter into a “closed” chromatin structure, evicted the transcription factor nuclear factor-1 from the promoter, and downregulated chromatin remodeling proteins and coregulatory molecules known to participate in the activation of the promoter-effects that occurred in the absence of histone acetylation of the local promoter chromatin structure.

The effects of HDACs depend upon the “cell context” which in turn influences acetylation or the interaction of HDACs with histone and nonhistone proteins. Ideally, comprehensive consideration of cell type, activity of HDAC isoforms and the nature of cell stimulation should be taken into account when effects of the inhibitor are examined in an experimental model. Lack of attention to these details can create an erroneous impression of contradictory results.

5. Future Perspectives

Current therapies for massive hemorrhage and severe sepsis largely focus on restoring tissue perfusion through resuscitation but have failed to address the specific cellular dysfunction caused by shock. Acetylation is rapidly emerging as a key mechanism that regulates the expression of numerous genes (epigenetic modulation through activation of nuclear histone proteins), as well as functions of multiple nonhistone proteins involved in key cellular functions such as cell survival, repair/healing, and anti-inflammation. HDACs hold great promise as a new class of agents for restoration of protein acetylation and treatment of hemorrhagic and septic shock.

Future studies should further elucidate the function of individual HDAC isoforms in severe hemorrhage and inflammation and assess potential effects of HDACs on sepsis following hemorrhagic shock. Since individual HDAC isoforms have distinctive physiological functions, it is important to develop next generation of HDACs. The new HDACs could then target specific HDAC isoforms and presumably would result in improved efficacy relative to the first generation pan inhibitors such as SAHA and TSA but with little adverse effects. In addition to being used as pro-survival agents for severe trauma hemorrhage, HDACs could be used as the combined pro-survival and anti-inflammatory drugs to prevent hosts from sepsis and even to treat sepsis following hemorrhage.

6. Summary

Experimental evidence has shown that treatment with HDACs increases endurance of animals subjected to lethal blood loss. The survival benefit is seen even when the drugs are administered postinsult, and is reproducible in different species including large animal models of polytrauma.

Protective properties of HDACs are not limited to hemorrhagic shock; HDAC treatment can also improve survival in LPS models of septic shock. Administration of HDACs modulates the immune system to create a favorable phenotype not only during the acute phase of hemorrhagic shock but also later when the septic complications are likely to occur. Repeated successes of HDACs in well-designed animal models of hemorrhagic shock (small and large animals) and septic shock (pre- and postshock treatments) suggest that modulation of protein acetylation is potentially a very useful strategy for the treatment of these critical diseases.

Abbreviations

ASK1:	Apoptosis signal regulating kinase 1
BAD:	Bcl-xl/Bcl-2 associated death promoter
Bcl-2:	B-cell lymphoma 2
β_2 -AR:	Beta2-adrenergic receptor
BMP7:	Bone morphogenetic protein 7
CBP:	Cyclic AMP (cAMP) response element binding protein (CREBP) binding protein
CINC:	Cytokine-induced neutrophil chemoattractant
DAMPs:	Damage-associated molecular patterns
DNA:	Deoxyribonucleic acid
DUSP5:	Dual specificity protein phosphatase 5
ELISA:	Enzyme-linked immunosorbent assay
ER:	Endoplasmic reticulum
ERK:	Extracellular signal regulated kinase
F-actin:	Filamentous actin
FWB:	Fresh whole blood
GSK-3 β :	Glycogen synthase kinase-3 β
H:	Histone
HATs:	Histone acetylases
HDA1:	Histone deacetylase A1
HDACs:	Histone deacetylases
HDACIs:	Histone deacetylase inhibitors
HMGB1:	High mobility group box 1
HS:	Hemorrhagic shock
Hsp 70:	Heat shock protein 70
Hsp 90:	Heat shock protein 90
IFN:	Interferon
IGF-1:	Insulin-like growth factor 1
IKK:	I κ B kinase
IRAK 1:	Interleukin-1 receptor associated kinase 1
IRF3:	Interferon regulatory factor 3
IV:	Intravenous (injection into a vein)
JNK:	c-Jun N-terminal kinase
LPS:	Lipopolysaccharide
MAGUK:	Membrane-associated guanylate kinase
MAPK:	Mitogen-activated protein kinase
MCP-1:	Macrophage chemoattractant protein 1
MEF2:	Myocyte enhancer factor 2
MKP-1:	MAP kinase phosphatase 1
MMTV:	Mouse mammary tumor virus
MODS:	Multiorgan dysfunction syndrome
MPO:	Myeloperoxidase
MSK1:	Mitogen and stress-activated protein kinase 1
MyD88:	Myeloid differentiation factor 88

NAD: Nicotinamide adenine dinucleotide
 NF- κ B: Nuclear factor kappa B
 PAMPs: Pathogen-associated molecular patterns
 PCAF: p300/CREB-binding protein-associated factor
 p300: p300 histone acetyl transferase
 PGC-1 α : Peroxisome proliferator-activated receptor γ coactivator-1 α
 PI3K: Phosphoinositide 3 kinase
 PIP2: Phosphatidylinositol 4,5-bisphosphate
 PIP3: phosphatidylinositol 3,4,5-triphosphate
 PKB: Protein kinase B
 PTEN: Phosphatase and tensin homolog
 ROS: Reactive oxygen species
 RSK2: Ribosomal S6 kinase 2
 SAHA: Suberoylanilide hydroxamic acid
 SIRS: Systemic inflammatory response syndrome
 SIRT: Sirtuins
 SMA: Superior mesenteric artery
 RT-PCR: Reverse transcription polymerase chain reaction
 TBP2: Trx binding protein 2
 TFs: Transcription factors
 TJ: Tight junction
 TLR4: Toll-like receptor 4
 TNF- α : Tumor necrosis factor α
 TRAF6: TNF receptor associated factor 6
 TRB3: Tribbles 3
 Trx: Thioredoxin
 TSA: Trichostatin A
 VPA: Valproic acid
 VSMCs: Vascular smooth muscle cells
 WT: Wild type.

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Review Article

The Role of HDACs Inhibitors in Childhood and Adolescence Acute Leukemias

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Acute leukemia is the most common type of childhood and adolescence cancer, characterized by clonal proliferation of variably differentiated myeloid or lymphoid precursors. Recent insights into the molecular pathogenesis of leukemia have shown that epigenetic modifications, such as deacetylation of histones and DNA methylation, play crucial roles in leukemogenesis, by transcriptional silencing of critical genes. Histone deacetylases (HDACs) are potential targets in the treatment of leukaemia, and, as a consequence, inhibitors of HDACs (HDIs) are being studied for therapeutic purposes. HDIs promote or enhance several different anticancer mechanisms, such as apoptosis, cell cycle arrest, and cellular differentiation and, therefore, are in evidence as promising treatment for children and adolescents with acute leukemia, in monotherapy or in association with other anticancer drugs. Here we review the main preclinical and clinical studies regarding the use of HDIs in treating childhood and adolescence leukemia.

1. Introduction

Acute leukemia is a malignancy characterized by clonal proliferation of variably differentiated myeloid or lymphoid precursors. It represents the most common type of childhood and adolescence cancer, accounting for 32% of all tumours diagnosed in children under 15 years of age and 26% of those diagnosed in adolescents under 20 years [1]. Over the past few decades, the prognosis of children and adolescents with acute leukemia has greatly improved, because of advances in risk assessment, tailored chemotherapy, optimal recourse to hematopoietic stem cell transplantation and innovative supportive care [2]. The cure rates now exceed 80% for children with acute lymphoblastic leukemia (ALL) and 50% for those with acute myeloid leukemia (AML) [3–5]. However, up to 20% of children with ALL and even more children with AML relapse, and these patients eventually present poor clinical outcome [6, 7]. Moreover a large number of patients presents several toxic side effects resulting from the very intensive chemotherapy [8]. Thus, novel therapeutic approaches are needed to increase the cure rate and the life's quality of these young patients.

In the past decade the increased understanding of the biology of acute leukemia, together with the implementation of high-throughput genomic techniques, have led to the development of molecular targeted therapies [9]. Epigenetic modifications, such as deacetylation of histones and DNA methylation, play crucial roles in the pathogenesis of many cancers including leukemia, by transcriptional silencing of critical genes [10]. The acetylation status of histones influences the chromatin conformation and consequently the transcription of genes. In normal cells there is a fine balance between acetylation and deacetylation of histones, depending on the activity of histone acetyl-transferases and histone-deacetylase (HDAC) [11] being the alteration of these enzymes possibly associated with tumorigenesis [12]. There are 18 HDACs in humans, 11 are zinc-dependent and fall into 4 classes on the basis of homology to yeast HDACs; the others are not zinc-dependent and not inhibited by compounds that inhibit zinc-dependent deacetylases [13]. HDACs has been reported in association with acute promyelocytic leukemia (APL) [14], and subsequently with many other hematologic and solid tumours [15]. Several studies have shown aberrant recruitment of HDAC-containing transcriptional repressor

complexes by the fusion proteins PML-RAR α or PLZF-RAR α and AML1-ETO in APL and AML, respectively, [16–19]. Furthermore, the constitutive upregulation of HOX genes that characterized AML with the mutation of the MLL gene has been shown to involve HDACs [20]. These observations led to the identification of HDACs as potential targets in the treatment of leukaemia. As a consequence, inhibitors of HDACs (HDIs) are being studied for therapeutic purposes, in an effort to upregulate the expression of the epigenetically silenced genes, potentially modifying the leukemic phenotype. HDIs promote apoptosis, cell cycle arrest and cellular differentiation, preventing malignant transformation [21, 22]. They are divided into several structural classes including short-chain fatty acids (such as valproic acid and butyrates), hydroximates (such as vorinostat and trichostatin-A), cyclic tetrapeptides (such as trapoxin and depsipeptide), benzamides (such as MS-275) and many other compounds [23]. In the last few years, studies of HDIs have shown that histone hyperacetylation can be achieved safely in humans [21]. Thanks to the promising initial results obtained in adults with myelodysplastic syndrome and acute leukemia [24, 25], clinical trials are starting to involve pediatric patients too. In the future the use of HDIs, in monotherapy or in association with other anticancer drugs, could be a promising treatment for children and adolescents with acute leukemia. Here we review the main preclinical and clinical studies regarding the use of HDIs in treating childhood and adolescence leukemia.

2. Preclinical and Clinical Trials

Although only a few targeted agents are currently in general use, the number of preclinical and clinical studies of HDIs continue to grow. The trials of HDIs involving children and adolescents with acute leukemia are reported below. Molecule's structures, chemical class of the molecules and development status are resumed in Table 1.

Vorinostat (Suberoylamilide Hydroxamic Acid, SAHA). It is a hydroxamic acid multi-HDI that blocks the enzymatic activity of both Class I (HDAC1, -2, and -3) and Class II (HDAC6) HDACs at low nanomolar concentrations (IC₅₀ < 86 nM) by directly binding to the catalytic site of these enzymes [26]. The anticancer potential of vorinostat was first demonstrated in nude mice transplanted with human prostate tumors [27]. Recently it has been approved by the U.S. Food and Drug Administration for the treatment of Cutaneous T-Cell Lymphoma (CTCL) and is now being evaluated also in other hematological malignancies [28].

Through its inhibitory action on HDACs, vorinostat has numerous effects on biological processes including cell cycle progression, apoptosis and differentiation at the cellular level, as well as angiogenesis inhibition and immune response modulation at the tissue level [26]. One model for the antitumor action of SAHA is that its inhibition of HDAC activity, and subsequent accumulation of acetylated histones, leads to the activation of genes whose expression causes induction of differentiation or apoptosis, thus inhibiting tumor growth. This model is based on the finding that

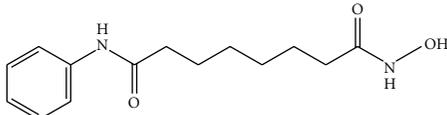
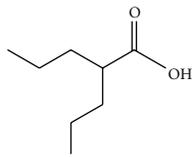
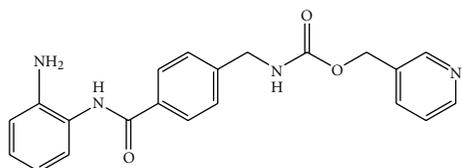
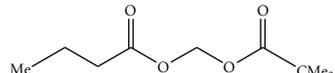
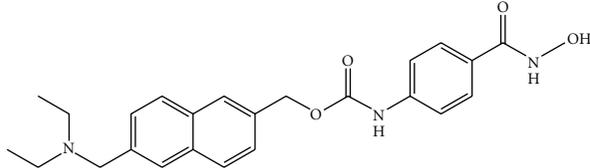
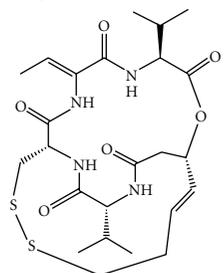
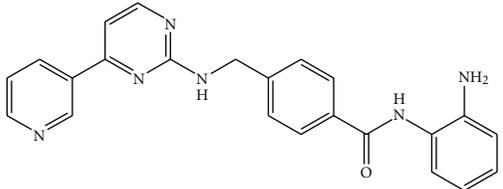
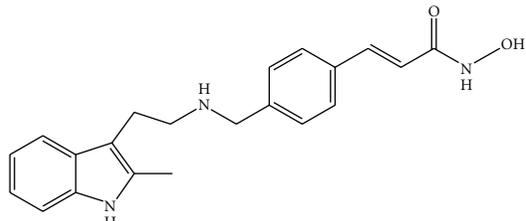
the expression of a relatively small number of genes (2–10% of expressed genes) is regulated following exposure of tumor cells to vorinostat [26]. One of the most commonly induced genes is the cyclin-dependent kinase inhibitor p21. However, rather than promoting apoptosis in tumor cells, it appears that vorinostat induced expression of p21 causes a cell cycle arrest in a p53-independent manner [29]. Moreover, increased acetylation of transcription factors such as p53, HIF-1 α , and E2F and increased acetylation of cytoplasmic proteins such as α -tubulin, cortactin, and HSP90 also contribute to vorinostat-induced cell cycle arrest, induction of cell death, and the inhibition of tumor growth [26].

Using two NOD/SCID mouse models, Einsiedel et al. demonstrated *in vivo* the efficacy of vorinostat in B-cell precursors childhood ALL. In fact, vorinostat was administered in a dose of 50, 100 or 150 mg/kg, in both subcutaneous and intravenous models, for 21 days and caused a clear growth suppression of the xenograft tumors [30].

As a multi-HDI, vorinostat may be well-suited for combination with other antineoplastic agents and radiation therapy, due both to its relatively low toxicity to normal cells and to its specific biological effects on cellular processes. Studies of the activity of vorinostat in combination with other cancer therapies (including radiation, kinase inhibitors, cytotoxic agents, and differentiating agents) have shown synergistic and additive activity in a variety of cultured human transformed cell lines [26]. Leclerc et al. showed that coadministration of SAHA plus the highly effective antileukemic drug methotrexate (MTX) synergizes to induce apoptotic death in ALL cells. When used as single agents, both MTX and SAHA can induce cell death, growth arrest and apoptosis, furthermore MTX plus SAHA synergistically increase apoptotic cell death and decrease viability in all B-precursor-ALL (SupB15, REH, NALM6, RCH-ACV) and T-ALL (CCRF-CEM) cell models tested [31]. Moreover, *in vitro* studies showed that SAHA causes growth inhibition, apoptosis, and potentiates retinoic acid induced differentiation in APL cells harboring t(15;17) (NB4 cell line) and in BM cells from PLZF-RAR α /RAR α -PLZF leukemic mice [32]. In the same study, He et al. demonstrated for the first time *in vivo* that SAHA induces an accumulation of acetylated histones at a nontoxic dose and, in combination with RA, prolongs survival and induces complete remission in APL transgenic mice refractory to retinoid acid treatment [32].

A phase I study of vorinostat in adult patients with leukemias was recently reported [33]. Significant antileukemia activity was observed in patients with AML, and vorinostat effectively inhibited HDAC activity in peripheral blood and bone marrow blasts. Further phase I and II trials of vorinostat as monotherapy or in combination regimens in adult AML are ongoing [33]. Moreover, a phase I study of vorinostat in pediatric patients with leukemia or solid tumors has just been published [34]. The primary purposes of this trial were to study the pharmacokinetics, the maximum tolerated dose (MTD) and the side effects of vorinostat in young patients (from 1 to 21 years old) and to determine the antitumor activity of SAHA administered with or without 13-cis retinoic acid in these patients.

TABLE 1: Main HDIs evaluated as emerging drugs for acute leukemias in pediatric and adult patients.

Compound	Structure	Chemical class	Development status
Vorinostat (SAHA)		Hydroxamic acid	Phase II
Valproic Acid		Short-chain fatty acid	Phase II
Entinostat (MS-275)		Benzamide	Preclinical and Phase I only in adults
Pivaloyloxymethyl butyrate (AN-9)		Butyric acid	Preclinical
Givinostat (ITF2357)		Hydroxamic acid	Preclinical
Depsipeptide or Romidepsin (FK228)		Cyclic tetrapeptide	Phase I only in adults
Mocetinostat (MGCD0103)		Benzamide	Phase I only in adults
Panobinostat (LBH589)		Hydroxamic acid	Phase I only in adults

This pediatric trial established the MTD of vorinostat as 230 mg/m²/d administered orally in patients with recurrent or refractory solid tumors, while patients with refractory leukemia did not appear to tolerate this dose due to liver dysfunction. Overall the authors pointed out that vorinostat

drug disposition and tolerance in children was similar to that observed in adult patients. Furthermore, other two phase II studies are reported as currently recruiting participants. The first [35] is a trial studying the efficacy of the use of decitabine and vorinostat together with combination chemotherapy

in treating patients with relapsed/refractory ALL or lymphoblastic lymphoma. They are recruiting patients from 2 to 60 years of age. The second study [36] is a clinical research study whose aim is to find the highest safe dose of vorinostat that can be given in combination with idarubicin and ara-C for the treatment of AML and high-risk MDS. They are recruiting patients from 15 to 65 years of age.

Valproic Acid (VPA). It is a short-chain fatty acid that is a weak HDI, but its long term availability as an antiepileptic drug prompted its evaluation in oncology as an epigenetic acting drug [28]. VPA can induce *in vitro* differentiation of primary AML blasts [37] and seems to exert its anticancer activity by inducing proteosomal degradation of HDAC, in particular of HDAC2 [38]. Furthermore, in t(8;21) AML, VPA might target AML1/ETO-driven leukemogenesis through the disruption of aberrant HDAC1 function [19]. In fact, it has been reported that VPA treatment disrupts the AML1/ETO-HDAC1 physical interaction, stimulates the global dissociation of AML1/ETO-HDAC1 complex from the promoter of AML1/ETO target genes, and induces a significant inhibition of HDAC activity, histone H3 and H4 hyperacetylation, and recruitment of RNA polymerase II, leading to transcriptional reactivation of target genes otherwise silenced by AML1/ETO fusion protein. These pharmacological effects resulted in significant antileukemic activity mediated by partial cell differentiation and caspase-dependent apoptosis [19]. Tonelli et al. showed that VPA induces strong cell growth inhibition in MLL-AF9 AML-M5 cells (THP-1, MM6 and MOLM-13) and AML-M5 blasts from a patient with a MLL gene rearrangement (MLL-mut). This *in vitro* study indicated that VPA could activate a p53-independent G1 cell-cycle arrest and apoptosis in MLL-mut AML [39]. Also Einsiedel et al. demonstrated that VPA inhibits proliferation, induces apoptosis and histone H4 hyperacetylation in BCP-ALL cell lines (Reh, Nalm6, Z33) *in vitro* [30]. Moreover they showed that VPA administration reduce the tumor growth significantly in two NOD/SCID mouse models of BCP-ALL. VPA treatment was able to inhibit the leukemia-induced splenomegaly of animals after intravenous challenge with ALL blasts, while no apparent toxicity was detected [30]. Studies in mice with PML-RAR α -induced leukemia demonstrated that VPA treatment causes upregulation of the death receptor proteins, TNF-related apoptosis inducing ligand (TRAIL), Fas, Fas ligand and DR5 in cells treated with VPA [40]. Also primary human AML samples that expressed neither PML-RAR α nor AML-ETO showed sensitivity to VPA and had increased apoptosis with evidence of upregulated TRAIL, DR5, Fas and Fas ligand levels in several cases that responded to HDAC therapy [40].

Several studies *in vitro* and *in vivo* showed that VPA is more efficacious in combination with other agents. Siitonen et al. investigated the effects of VPA as a single agent and in combination with cytarabine (Ara-C) and etoposide in an acute myeloblastic cell line (OCI/AML-2). They observed a dramatic increase in cytotoxicity combining VPA with Ara-C, whereas coaddition with etoposide had a much smaller effect on cell death. In addition, VPA induced

a clear G1 phase arrest and up-regulated cyclin D1 expression in the presence of Ara-C and etoposide [41]. Sanchez-Gonzalez et al. studied the cellular and molecular effects of combining the anthracycline idarubicin with VPA in HL-60 and MOLT4 cell lines [42]. Their results indicate that the combination of an anthracycline with an HDI displays a synergistic effect *in vitro* and should have significant clinical activity in patients with leukemia [42]. Antileukemia activity has also been evaluated with VPA administered in combination with hypomethylating agents. The combination of 5-azacitidine (5-AZA), a hypomethylating agent with significant antileukemia activity in humans, with VPA was studied in the leukemic cell lines HL-60 and MOLT4. It showed a synergistic effect in growth inhibition, induction of apoptosis, and reactivation of protein p57 and p21 on human leukemia cell lines, suggesting that the combination of DAC and VPA could have significant antileukemia activity *in vivo* [43]. Several phase I/II studies of VPA monotherapy or in combination have been completed in adult patients. They include VPA plus decitabine [25], VPA plus retinoic acid (ATRA) [44, 45], and VPA plus 5-AZA and ATRA [46]. Garcia-Manero et al. conducted a phase 1/2 study of the combination of decitabine and VPA in 54 patients with advanced leukemia and a median age at diagnosis of 60 years [25]. An objective response was observed in 22% patients, including 19% with a complete remission, and the overall survival was 15.3 months. This study enrolled also seven pediatric patients aged 4 to 21 years affected by relapsed/refractory AML. Although neither complete nor partial remission was described, 3 patients achieved a complete marrow response, and 1 patient presented only 6% of marrow blasts. No pediatric cases presented significant toxicities related to therapy [25]. Soriano et al. conducted a phase 1/2 study of the combination of 5-AZA, VPA and ATRA in patients with AML or high-risk myelodysplastic syndrome [46]. The trial involved a total of 53 patients with a median age at diagnosis of 69 years. The combination of the drugs resulted safe and active, with an overall response rate of 42% and a median remission duration of 26 weeks. In their trial, Soriano and colleagues involved also 3 pediatric patients with recurring/refractory AML, and the combination of 5-AZA, VPA and ATRA led to BM response in 1 case [46]. Thus, combinations of VPA with 5-AZA or decitabine and ATRA need to be further explored in specific pediatric phase 1 studies in leukemia.

Entinostat (MS-275 or SNDX-275). It is a novel and orally available synthetic benzamide HDI that preferentially inhibits HDAC1, but does not possess activity against HDAC6 [47]. Rosato et al. conducted a study on MS-275 in human leukemia cell lines (U937, HL-60, Jurkat, and K562) and leukemic blasts obtained from the peripheral blood of patients with AML. They demonstrated that MS-275 exerts dose-dependent effects on human leukemia cells: a p21-dependent growth arrest and differentiation at low drug concentrations and a marked induction of ROS, mitochondrial damage, caspase activation, and apoptosis at higher concentrations [48]. One study explored

the effect of MS-275 against a panel of leukemia cells of human origin, each with defined genetic alterations. MS-275 significantly induced growth arrest and apoptosis in the AML cell line, MOLM13, and in the biphenotypic leukemia cell line, MV4-11, which both possess an internal tandem duplication mutation in the FLT3 gene. MS-275 induced acetylation of HSP90 and ubiquitination of FLT3, which hesitated in the degradation of FLT3 via the proteasome pathway. Moreover, this study found that further inhibition of MEK/ERK signaling potentiated the effects of MS-275 in these cells. These results suggest that MS-275 may be useful in treating leukemic patients with a mutation on the FLT3 gene [49]. Another recent study showed that MS-275 blocks Akt/mammalian target of rapamycin (mTOR) signaling in AML HL60 and APL NB4 cells, inducing growth arrest and differentiation. In addition, inactivation of mTOR by rapamycin analog RAD001 (everolimus) significantly enhanced MS-275-mediated growth inhibition and apoptosis of these cells and potentiated the ability of MS-275 to induce the differentiation of HL60 and NB4 cells. These results suggest that concomitant administration of an HDI and an mTOR inhibitor may be a promising treatment strategy for patients with AML or APL [50]. Moreover, MS-275 might be used to enhance the antileukemic activity of established nucleoside analogues such as fludarabine. In fact Maggio et al. examined the interaction between MS-275 and fludarabine in lymphoid and myeloid human leukemia cells and demonstrated that sequential treatment of Jurkat lymphoblastic leukemia cells with MS-275 and fludarabine induces mitochondrial injury, caspase activation, and apoptosis [51]. The antitumor efficacy of MS-275 was also evaluated *in vitro* in a series of pediatric solid tumor cell lines, including neuroblastoma, rhabdomyosarcoma, Ewing's sarcoma (EWS), retinoblastoma, medulloblastoma, undifferentiated sarcoma (US), osteosarcoma, and malignant rhabdoid tumors and *in vivo* using xenograft orthotopic models of US, EWS, and neuroblastoma [52]. Jaboin et al. showed that MS-275 treatment of pediatric tumor cell lines induced the expression of p21 mRNA and different effects on cell cycle, like apoptosis, while drug administration in mice inhibited the growth of established tumors. Moreover, a phase I and pharmacologic study of MS-275 in adults with refractory and relapsed acute leukemias was reported by Gojo et al. [53]. They observed that the maximum-tolerated dose was 8 mg/m² weekly for 4 weeks every 6 weeks and dose-limiting toxicities included infections and neurologic toxicity manifesting as unsteady gait and somnolence. They demonstrated that MS-275 effectively inhibits HDAC *in vivo* in patients, in fact the treatment induced an increase in protein and histone H3/H4 acetylation, p21 expression, and caspase-3 activation in bone marrow mononuclear cells.

Pivaloyloxymethyl Butyrate (AN-9). It is a relatively new member of an established family of acyloxyalkyl ester prodrugs of carboxylic acids that undergo rapid hydrolysis and its anticancer effect is assumed to stem primarily from the release of butyric acid [54]. AN-9 induces acetylation of histones at a concentration one order of magnitude

lower than butyric acid *in vitro* and this is most likely due to its increased permeability across cell membranes [55]. Batova et al. investigated the *in vitro* therapeutic efficacy of AN-9 in HL-60 cell line and primary human acute leukemia cells isolated from bone marrow and the peripheral blood of pediatric patients with AML or B-precursor ALL, including doxorubicin-resistant and/or clinically refractory acute leukemias [54]. They demonstrated that AN-9 has antiproliferative and cytotoxic effects on all the leukemia cells tested, including a doxorubicin-resistant T-ALL, a clinically refractory relapsed AML, and a relapsed infant ALL characterized by an 11q23 rearrangement and a very poor prognosis. Furthermore, AN-9 is less toxic to normal hematopoietic progenitors and thus has selectivity for leukemia cells, indicating that AN-9 is a selective anticancer agent. The mechanism behind the antiproliferative effect of AN-9 appeared to be generally p21-independent and the increased apoptosis was thought to be mediated through the reduction in expression of the antiapoptotic bcl2 gene or alternatively through the induction of genes involved in the death receptor pathway [54]. Additionally, another advantage of AN-9 is its ability to synergize with anthracyclines such as daunomycin, commonly used in the treatment of leukemia patients, allowing the use of a lower dose of this drug and subsequently less toxicity. In fact Kasukabe et al. demonstrated that AN-9 is an effective antitumor agent in mouse monocytic leukaemia Mm-A cells and in human myeloid leukemia cell lines and that the administration of AN-9 plus daunorubicin markedly prolongs the survival of mice inoculated with Mm-A cells [56]. These several studies demonstrated the selective toxicity of AN-9 to acute leukemias, including drug-resistant relapsed leukemias, and thus provide the rationale for the initiation of clinical trials of AN-9 in acute leukemias of both adults and children. Furthermore three clinical trials of AN-9 in adults with others hematologic and solid tumours have been terminated, but the results have not yet been published. The first is a phase II study to assess the safety and efficacy of AN-9 in adult patients with chronic lymphocytic leukemia who have relapsed or refractory disease after previous chemotherapy treatment [57]. The second is a pilot phase I and II study of AN-9 in adult patients with malignant melanoma who have relapsed after treatment with chemotherapy or Interleukin-2 [58]. The last is a phase II comparative trial of AN-9 and Docetaxel versus Docetaxel monotherapy in adult patients with advanced nonsmall cell lung cancer [59].

Givinostat (ITF2357). It is a synthesized HDI containing a hydroxamic acid moiety linked to an aromatic ring. It has been shown to inhibit the autonomous proliferation of haematopoietic cells bearing the JAK2(V617F) mutation, through a specific downmodulation of the JAK2(V617F) protein and inhibition of its downstream signalling [60]. Recently a phase II study has been reported in adult patients with JAK2V617F positive chronic myeloproliferative neoplasms [61]. Rambaldi observed that ITF2357 at doses of 50 mg twice daily was well tolerated and could induce haematological response in most polycythemia vera and

some myelofibrosis in adult patients carrying the JAK2 V617F mutation. In addition a phase II study testing givinostat in combination with hydroxyurea in a population of adult patients with JAK2V617F positive polycythemia vera nonresponders to the MTD of hydroxyurea monotherapy for at least 3 months is recruiting patients [62]. Furthermore ITF2357 was tested using an AML cell line as a model and it showed selective low-dose antileukemic activity on AML1/ETO-positive cells, emerging as a potent therapeutic agent, particularly in AML1/ETO-positive cells [63].

Depsipeptide (Romidepsin, FK228). It is a cyclic peptide that selectively inhibits HDAC isotypes 1, 2, 4 and 6 [28]. Recently it has been approved by the U.S. Food and Drug Administration for the treatment of Cutaneous T-Cell Lymphoma (CTCL) [64, 65] and is now being evaluated also in other hematological malignancies. Preclinical studies demonstrated that *in vitro* it inhibits HDAC in human APL cell line NB4, furthermore *in vivo* administration of depsipeptide alone partly inhibited the growth of established tumors of NB4 subcutaneously transplanted in NOD/Shi-scid/scid mice and its combination with ATRA was synergistically effective in preventing leukemia death [66]. Depsipeptide was shown to inhibit HDAC *in vivo* in a phase I trial in adult patients with AML: several patients had evidence of antitumor activity following treatment, but no partial or complete responses were noted. Moreover its administration was limited by progressive adverse symptoms that prevented repeated dosing [67, 68]. Therefore depsipeptide monotherapy appears to have limited clinical activity both in adult and pediatric patients.

Mocetinostat (MGCD0103). It is an isotype-specific aminophenylbenzamide that inhibits HDAC isotypes 1, 2, 3 and 11 [69]. Preclinical studies have demonstrated that MGCD0103 is orally bioavailable with significant *in vitro* antineoplastic activity at submicromolar concentrations against a broad spectrum of human cancers, including various leukemia cell lines and xenografts [70]. A phase I study in adult patients with AML demonstrated a favorable safety profile and showed activity as a single agent leading to a complete bone marrow response [69].

Several other HDIs have shown promise in adults in early phase I or small phase II trials. The hydroxamate *panobinostat (LBH589)* has attractive preclinical and phase I safety and efficacy profiles, with evidence of activity in AML [71]. Scuto et al. investigated the mechanism of action of LBH589 in two Philadelphia chromosome-negative (Ph(-)) ALL cell lines (T-cell MOLT-4 and preB-cell Reh). Low nanomolar concentrations of LBH589 induced cell-cycle arrest, apoptosis, and histone hyperacetylation. LBH589 treatment also increased mRNA levels of proapoptosis, growth arrest, and DNA damage repair genes. This treatment was active against cultured primary Ph(-) ALL cells, including those from a relapsed patient, providing a rationale for exploring the clinical activity of LBH589 in treating patients with Ph(-) ALL [72]. A phase I study of LBH589 has been reported in adult patients with refractory

hematologic malignancies [73]. Giles et al. observed that intravenous administration of LBH589 was well tolerated at doses <11.5 mg/m², while higher doses induced cardiac and other toxicities like nausea, diarrhea, vomiting, hypokalemia, loss of appetite and thrombocytopenia. Overall the drug treatment showed consistent transient antileukemic and biological effects that would be further explored using the oral formulation of LBH589.

3. Conclusions and Perspectives

In recent years HDIs have shown potential anticancer activity against a variety of solid and hematologic tumors, included leukemia. Vorinostat and depsipeptide have already been approved by the U.S. Food and Drug Administration for the treatment of cutaneous T-cell lymphoma [28, 65], and the clinical use of HDIs in adults with leukemia may become real in a short time. Several *in vitro* and *in vivo* studies have been performed to test the role of HDIs in leukemia treatments. The data from the adult trials show that HDIs, in monotherapy as well as in combination therapy, are generally well tolerated. However, since there are very few patients who have been treated for a long time, it will be necessary to monitor long-term effects in the future. However, the available data support the rationale for using HDIs in the treatment of childhood and adolescence leukemia. In the last few years, clinical trials of these epigenetic modulators have started to involve also children and adolescents affected by acute leukemia [25, 46]. Currently phase I-II clinical trials are evaluating the efficacy of vorinostat and VPA in treatment of childhood and adolescent leukemia, alone or in combination with other therapeutic agents [34–36]. In conclusion, further clinical trials involving HDIs are needed to consolidate the clinical use of these agents in both adult and pediatric acute leukemia. Moreover further studies are needed to determine whether specific cytogenetic or molecularly defined subgroups can predict response or resistance to HDIs therapy.

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Review Article

Elongator: An Ancestral Complex Driving Transcription and Migration through Protein Acetylation

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Elongator is an evolutionary highly conserved complex. At least two of its cellular functions rely on the intrinsic lysine acetyltransferase activity of the Elongator complex. Its two known substrates—Histone H3 and α -Tubulin—reflect the different roles of Elongator in the cytosol and the nucleus. A picture seems to emerge in which nuclear Elongator could regulate the transcriptional elongation of a subset of stress-inducible genes through acetylation of Histone H3 in the promoter-distal gene body. In the cytosol, Elongator-mediated acetylation of α -Tubulin contributes to intracellular trafficking and cell migration. Defects in both functions of Elongator have been implicated in neurodegenerative disorders.

1. Introduction

Elongator was initially identified in yeast as part of the RNA polymerase II (RNAPII) holoenzyme [1]. Since this interaction was dependent on the hyperphosphorylation of the C-terminal repeats of RNAPII, which is a mark of transcriptional elongation, the complex was termed Elongator. The fully functional Elongator complex consists of six subunits [2–4] and has a physiologically important role. This is for instance demonstrated by several observations. First, loss of Elongator function in yeast provokes hypersensitivity to stresses including elevated temperature and osmotic conditions [2, 5]. Second, in *Arabidopsis*, mutations in Elongator result in growth defects [6]. Moreover, mice mutations in Elongator subunits are embryonic lethal [7], and finally, impaired Elongator activity has been correlated with familial dysautonomia in human patients [8].

Both yeast and human Elongator have lysine acetyltransferase (KAT) activity [5, 9, 10]. Only two main substrates for Elongator have been identified: Histone H3 and α -Tubulin. These two different substrates reflect the different functions of Elongator complex in the nucleus and in the cytosol. After having been identified in the context of transcription, it was first surprising to find that the major fraction of

the Elongator complex indeed resided in the cytosol [9–12]. While in the nucleus acetylation of Histone H3 is linked to the function of Elongator in transcription [13], cytosolic acetylation of α -Tubulin by Elongator has been recently connected to microtubules function particularly in the context of cell migration [14].

Other functions of Elongator have also been described. These include roles in tRNA processing [15, 16] and exocytosis [17], which have been reviewed elsewhere [13, 18]. Here, we will focus on the Elongator functions for which the involvement of protein acetylation has been well established. In particular, we will discuss similarities and differences in its molecular mode of function in the nucleus and cytosol. We will further discuss the link between the dysfunction of the Elongator complex and neurological disorders.

2. Biochemical and Other Aspects of Elongator

Elongator was first copurified with yeast RNAPII as three subunits containing complex [1]. Direct affinity purifications, however, made clear that the functional unit of Elongator contains six subunits that can be separated in two stable sub-complexes under high-salt conditions [2–4].

The subunits of these two complexes were termed Elp1-3 and Elp4-6. Although these proteins have been given many other names depending on the contexts of their initial identification, for simplification, here we will stick to the Elp nomenclature.

All Elp proteins are evolutionary highly conserved which is best illustrated by cross-species rescue experiments. *Arabidopsis* Elp1 could rescue deficient yeast [19], and human Elp1 could rescue Elp1-deficient neurons in mouse cortex [7]. Human Elp3 and Elp4 could further compensate for the lack of their counterparts in yeast [20].

Within the Elongator complex, the 150 kDa protein Elp1 is the largest subunit. It contains several WD40 repeats and is considered to mainly function as scaffold protein, which is required for the formation of the complex [21]. More recently, however, Elp1 proteins from yeast, *C. elegans*, and human were shown to possess RNA-dependent RNA polymerase activity [22]. Elp2 also contains WD40 domain [23]. In contrast to Elp1, however, Elp2 does not seem to provide any scaffolding function since the remaining complex could form in its absence [24]. The Elp3 subunit of the complex possesses motifs characteristic of the GCN5 histone acetyl transferase family [5] and was shown to be able to acetylate histones [25]. In addition, Elp3 contains an iron-sulfur cluster, which can bind S-adenosylmethionine [26] and which is essential for the structural integrity of the Elongator complex [27]. It was first suggested that this domain could have catalytic activity and function as histone demethylase [28], but experimental studies failed to confirm this hypothesis. Instead, recent evidence suggests that Elongator might be involved in DNA demethylation [30]. The integrity of Elongator, and more particularly the iron-sulfur cluster of Elp3, was shown to be essential for the global zygotic DNA demethylation occurring on the paternal genome at the postfertilization stage [30]. On the basis of sequence comparisons, it was suggested that Elp4 and Elp6 could be inactive orthologues of ancestral ATPases involved in chromatin remodeling [31].

Combining genetic inactivation and affinity purifications [11, 21], Frohloff and colleagues were the first to put forward a model for the architecture of the hexameric Elongator complex that was largely confirmed by others [32]. While efforts to determine the three-dimensional structure of the Elongator complex are still under way, the model can serve as a good basis for our experimental design. As shown in Figure 1, Elp1 is the central subunit that mediates the binding of the two subcomplexes. On one side, Elp1 directly interacts with Elp3, which in turn binds Elp2. On the other hand, Elp1 also directly binds the Elp4-6 subcomplex through Elp4. Whether Elp6 and Elp5 also directly contact Elp1 is less clear at present.

The functional unit of Elongator is the holocomplex containing all six subunits. This is best illustrated in yeast where strains lacking any of the 6 Elp proteins have very similar phenotypes. For instance, they display a particular “slow start” phenotype as manifested by a pronounced delay in adaptation to new growth medium [1, 4]. Elp-deficient strains are further temperature, salt, and caffeine sensitive [2, 4] and resistant to the *K. lactis* killer toxin [3, 33]. There

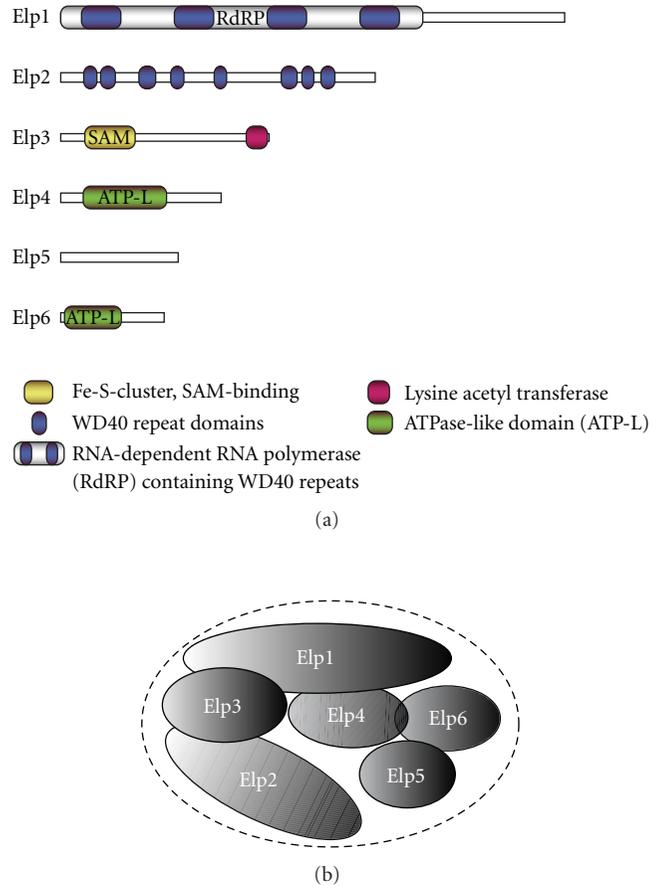


FIGURE 1: The Elongator complex and its components. (a) Schematic view of the Elp proteins and their domain structure. (b) Current model for the architecture of the Elongator complex.

is still some ambiguity in the questions whether elp-mutant strains are sensitive to the pyrimidine analogue 6-azauracil [1–3]. Most importantly, KAT activity of Elongator was essential for the rescue of all phenotypes in yeast. Removal of almost any of the Elongator subunits affects the interaction of the others [11, 21, 24, 32] and might explain why all subunits are functionally essential *in vivo*. In line with this, it could be demonstrated that Elongator requires all its subunits for efficient KAT activity [34].

Several proteins have been reported to interact with components of the Elongator complex. These include Kti12 [33], IKB [35, 36], STAT3 [36], and PCNA [32]. Although some interactions have been confirmed in different species, most have only been found in a single species. It remains to be seen which interactions are as conserved as Elongator itself and which have evolved later. Mainly from studies focusing on zymocin resistance in yeast, for instance, comes the statement that Elongator interacts with the Hrr kinase and the Sit4 phosphatase [37, 38]. In a series of publications, the authors establish that these interactions result in cycles of phosphorylation and dephosphorylation events that are essential for Elongator function in zymocin resistance. Whether such a phosphorylation-dependent control is also

relevant for the other functions of Elongator remains to be elucidated. In addition, several interactions that have been found by one group could so far not be confirmed by others, which suggests that many of these interactions could be transient and might thus only occur under distinct conditions. These “potential” interactors are not further discussed here.

3. Acetylation of Histones: Elongator in Transcription

A surprising large number of genes, which can be induced by external stimuli, are regulated at the level of transcriptional elongation [39, 40]. These genes include stress-inducible genes as well as genes activated during developmental processes. For these genes, RNAPII is stalled or paused in close proximity to the promoters in the absence of the stimuli. Upon stimulation, the C-terminal domain of RNAPII becomes hyperphosphorylated and resumes transcription whose procession is controlled by elongation factors. These elongation factors can be divided into active and passive factors in respect to whether or not they directly affect enzymatic activity of RNAPII [41]. After several years of controversy that followed the initial copurification of the Elongator with transcription-competent RNAPII [1], today there is little doubt that one of the functions of Elongator is indeed transcriptional elongation. However, the exact mode of action is still not fully understood. Also the debate whether the Elongator should be considered as active or passive elongation factor is still open. Here, we review the data of the last ten years that link Elongator to transcriptional elongation.

The observation that Elp3 subunit is similar to the GCN5 suggested early that the Elongator complex could be a histone acetyltransferase [5]. *In vitro*, purified Elp3 was able to acetylate both H3 and H4 [5], and *in vivo*, a mutant lacking its putative KAT activity had a similar phenotype as complete loss of Elp3 [25, 33]. Synthetic lethality experiments further provided the first evidence pointing towards Histone H3 and in particular Lysine 14 as main *in vivo* target of Elp3 [25]. The functionality of Elongator’s KAT activity and its specificity for Histone H3 were later confirmed in several species including yeast, plants, and mammalian cells [34, 42, 43]. *In vitro*, Elongator was able to enhance RNAPII transcription from a chromatinic template in an Acetyl-CoA-dependent manner [10], while, on naked DNA, Elongator had no influence on RNAPII transcription [2, 10].

The target genes of Elongator depend a lot on the biologic system studied. In yeast, loss of Elongator function reduced the activation of a number of inducible genes [1, 2, 5] but did not affect transcription globally [2]. In plants, Elongator contributes to the transcription of stress-induced and auxin-related genes [19, 43]. In human cells, Elongator was required for the activation of several genes involved in migration [42] and for the activation of HSP70 in heat shock conditions [29]. Elongator was also involved in regulation of several p53-dependent proapoptotic genes but in a manner that did not seem to occur on the transcriptional level [44].

In line with a possible function for Elongator in regulating these genes by facilitating their transcriptional elongation, several groups could now demonstrate that Elongator preferentially occupies the open reading frames but not the promoters of these genes [29, 42]. Indeed, Elongator binding was mostly observed several kilobases downstream of the transcriptional start site (TSS) [45]. In yeast, Elongator could be cross-linked to the nascent pre-mRNAs of regulated genes [46]. In line with the initial purification of Elongator with the hyperphosphorylated form of RNAPII [1], time-course experiments suggested that Elongator indeed arrives at genes after hyperphosphorylation of RNAPII [47]. It has to be pointed out that Elongator could also be found on genes whose transcription was not affected by its depletion suggesting some functional redundancy of Elongator with other elongation factors. Taking the function of Elongator in the context of chromatin into account, it is the most similar to the FACT complex. FACT was suggested to regulate Elongation by facilitating the procession of RNAPII through nucleosomes [41]. In human cells, both FACT and Elongator could be found on a common gene, where in contrast to Elongator, FACT was bound at the more TSS-proximal regions of the ORF [45]. This observation provokes the intriguing speculation that FACT and Elongator could have distinct functions in the early and late elongation process. It is further interesting to note that genetic inactivation of both FACT and Elongator resulted in synthetic lethality in yeast [48]. Whether Elongator facilitates the opening and reconstitution of nucleosomes in a FACT-like manner remains to be investigated.

Taken together, a picture emerges in which Elongator regulates the transcriptional elongation of a subset of stress-inducible genes through acetylation of Histone H3 in the TSS distal gene body (see Figure 2). Although the elongation function of Elongator is conserved, the subset of regulated genes has diverged during evolution. Many open questions remain such as how is the target gene specificity of Elongator determined? What is the molecular consequence of Elongator-mediated histone acetylation? Considering the other functions of Elongator, manipulation of its function is likely to affect indirect as well as direct target genes. Hence, it will inevitable to check for the presence of Elongator at putative target genes when addressing the role of Elongator in transcription.

4. Acetylation of Tubulin: Elongator and Cellular Motility

The cytoskeleton is characterized by its dynamic structure and is composed of three types of fibers: actin filaments, intermediate filaments, and microtubules [49]. The functions of the cytoskeleton go far beyond the maintenance of cell shape and include important roles in cellular motility, intracellular transport, and mitosis [50].

Microtubules are the biggest cytoskeletal fibers that are essentially composed of only two proteins, the α - and β -Tubulins [51]. Heterodimers of globular α - and β -Tubulins polymerize in a head-to-tail fashion to form the so-called

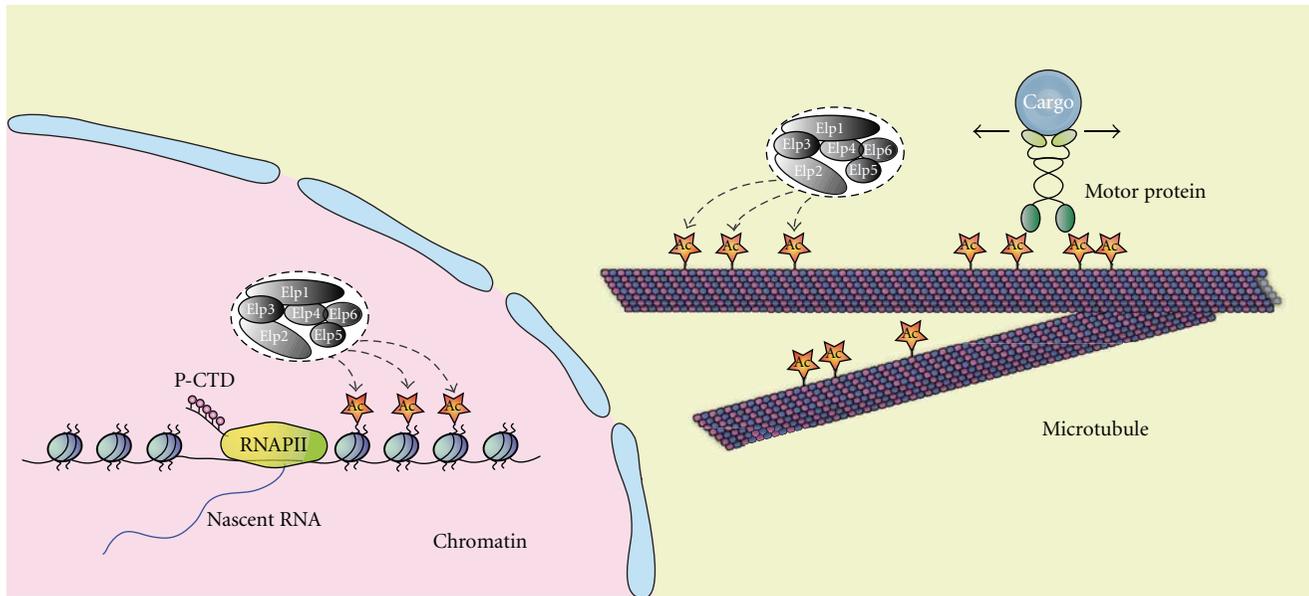


FIGURE 2: Two acetylation-dependent functions of Elongator. In the nucleus, Elongator-mediated acetylation of Histone H3 contributes to transcriptional elongation. Thereby, it interacts with processive RNAPII, which is hyperphosphorylated at its C-terminal domain (CTD). Although this function of Elongator is conserved from yeast to man, the subset of genes that underly this kind of regulation have diverged during evolution. In the cytosol, Elongator acetylates the α -Tubulin subunit of microtubules. This acetylation event contributes to the trafficking of cargo along microtubules and cell migration. Please note that acetylation of α -Tubulin occurs in the lumen of microtubule, but for reasons of illustration, it has been depicted on outside here.

protofilaments. Of these protofilaments, 13 bind to each other in a parallel way to form the tubular structure of the microtubule, which has a diameter of about 25 nm. An important characteristic of these microtubules is their polarity. The early *in vitro* observation that new heterodimers added faster to one end of the microtubules leads to the distinction of plus and minus ends [52, 53]. Minus ends terminate in α -Tubulin subunits and plus-ends accordingly in β -Tubulin subunits. By establishing and maintaining cell polarity, the remodelling of microtubules is particularly important in directed cell migration processes [54, 55].

Several PTMs occur on Tubulins that are considered to modulate the function of microtubules by providing binding sites for regulatory proteins [56]. These PTMs include tyrosination, polyglycylation, and polyglutamylation. Considering the tubular architecture of microtubuli it is not surprising that most PTMs occur on the carboxyterminal domains of both α - and β -Tubulins, which are exposed on the outer surface of the tube [57].

Additionally, α -Tubulin but not β -Tubulin is acetylated *in vivo* [58]. The particularity of this PTM is its localization. The acetylation of α -Tubulin, which exclusively occurs on lysine 40, is located on the luminal inner surface of the microtubule [59]. While HDAC6 and Sirt2 have been described a few years ago as tubulin deacetylases [60–62], the responsible acetylase remained elusive. Just recently, the Elp3 subunit of cytosolic Elongator was identified as α -Tubulin-specific KAT in human and mouse cells [14]. It could be shown by mutating a critical residue in the catalytic domain of Elp3 that Elongator is responsible for a substantial

amount of tubulin acetylation [14]. Importantly, acetylation of tubulin by Elongator does also occur in the nematode *C. elegans* [63] suggesting a similar high degree of conservation for the cytosolic function of Elongator as previously shown for its nuclear role in transcription. The identification of MEC-17 as a second α -Tubulin acetyltransferase made clear that Elongator is not the only one [64]. Indeed, in *Tetrahymena* and neurons from zebrafish embryos and *C. elegans* hermaphrodites, MEC-17 was shown to be required for most if not all α -Tubulin acetylation. We can expect additional acetyltransferases to be identified in the future, and the task before the scientific community will be to sort out the context-dependent contributions of each of these enzymes.

Acetylation of microtubules seems not to be essential for cell survival but plays a role in cell motility and in motor-based trafficking [65, 66]. The role of acetylation in cell motility was first demonstrated by several studies reporting that HDAC6 overexpression that promotes α -Tubulin deacetylation increases cell motility whereas HDAC6 inhibition triggers the opposite effect [60, 67]. The migration defects observed in neurons depleted for Elongator subunits confirmed the important function of tubulin acetylation during motility processes [14, 63]. Similarly, the loss of the other acetyltransferase MEC-17 reduced the touch sensitivity of neurons in *C. elegans* and induced phenotypes consistent with neuromuscular defects in zebrafish embryos [64].

However, what is the molecular mechanism by which acetylation of tubulin affects cellular motility? Some clues came from studies of motor proteins that transport diverse cargoes along microtubules. Microtubule-specific motor

proteins are divided into two classes called dyneins and kinesins depending on the direction of their movement [68, 69]. Most of the members of the kinesin superfamily move to the plus end of microtubules, whereas members of the dynein superfamily transport their cargoes to the minus end [70, 71].

Acetylation of α -Tubulin was shown to promote in particular the binding and the motility of kinesin-1 [66]. In the cerebral cortex, Elongator mediated the acetylation of microtubules and could thereby direct anterograde transport of defined cargoes to growing neurites in developing neurons and to axons in mature cells [18]. Acetylation of α -Tubulin was also associated recently to the posttranslational fine tuning of α -Tubulin levels and to the dynamics of polymerization and depolymerization [63]. Both transport and changes in the microtubule dynamics could directly influence the cellular motility.

With the identification of α -Tubulin as cytosolic target for the Elongator's KAT activity, a new chapter was opened. Many questions persist. For instance, it is not clear how Elongator or the counteracting deacetylases HDAC6 and SirtT2 can access lysine 40 at its luminal position. It is further still enigmatic how a luminal modification can influence the motor protein functions that occur on the cytoplasmic surface of the microtubule. How are the activities of α -Tubulin-specific acetylases regulated? It is interesting to point out that the level of α -Tubulin acetylation is quite variable. In neurons, for instance, the large majority of cellular α -Tubulin is acetylated.

5. Defects in Elongator and Neurodegenerative Disorders

Elongator is associated to different neuronal diseases. While the familial dysautonomia is associated to mutation of the *IKBKAP* gene encoding Elp1 [8, 72], the amyotrophic lateral sclerosis was recently shown to be linked to allelic variants of *ELP3* [73].

Familial dysautonomia (FD), also known as Riley-Day syndrome, is characterized by a progressive depletion of unmyelinated sensory and autonomic neurons resulting in a loss of neuronal function which leads to a complex symptomatology including gastrointestinal dysfunction, lack of overflow tears, absence of fungiform papillae on the tongue and other autonomic disturbances [74]. The major genetic cause of FD is a single noncoding mutation of the *Elp1* encoding gene which affects its splicing. Interestingly, this mutation is not fully penetrant, which means that some residual amounts of the wild-type *Elp1* (*Elp1*-WT) mRNA are nevertheless synthesized. The central nervous and the peripheral nervous systems are the major tissues affected by this splicing defect as demonstrated by the low level of WT-*Elp1* present in these tissues [75]. Amyotrophic lateral sclerosis (ALS) is caused by the degeneration of motor neurons resulting in muscle weakness and atrophy. Affected patients show cognitive impairments and usually die from respiratory muscle weakness. Defects in Elongator have already been implicated in the pathogenesis of ALS [73].

The observation that alterations of Elongator correlate with two different neurodegenerative disorders suggests that Elongator has an essential function in the development and the maintenance of neuronal networks. On the molecular level, Elongator exerts its "neuronal" function at least partially through acetylation of its two main substrates—Histone H3 and α -Tubulin. On one hand, defective Elongator could reduce the elongation of gene transcripts that normally mediate the motility, the development, and the survival of neuronal cells [42]. On the other hand, defects in intracellular trafficking have already been linked to several neurodegenerative diseases including Alzheimer's disease, Parkinson, disease, or ALS [76–78]. Thus, it is reasonable to speculate that the loss of Elongator-mediated tubulin-acetylation could result in defective intracellular transport of cargo and thereby contribute to neurodegeneration, but we have also to point out that Elongator might have other yet unknown substrates that could contribute to the symptomatology of FD and ALS.

6. Final Considerations and Outlook

The two substrates of Elongator's acetylase activity— α -Tubulin and Histone H3—are as different as the functions of Elongator in the cytosol and the nucleus, or, do they have anything in common? Well, both α -Tubulin and Histone H3 are main components of cellular macrocomplexes: microtubules and chromatin. Both microtubules and chromatin are formed by repetitive units. We can speculate that Elongator might act as a processive enzyme in which each acetylation reaction would facilitate the next. This would predict that Elongator has a preference for carrying out sequential reactions. Experimentally, this could be tested in acetylation assays by comparing single tubulin dimers and microtubules. For both microtubules and chromatin it is evident that Elongator is not the only acetylating enzyme. In both cases acetylation has further been suggested to be one of many modifications that together could form a type of code. According to the histone or tubulin code hypotheses [79, 80], the combination of modifications would be read and interpret by factors that ultimately regulate the function of chromatin and microtubules, respectively.

It was known that Elongator has also other acetylation-independent functions in secretion and tRNA processing [13]. Recent evidences further linked Elongator to the DNA-damage response and gene silencing [32], RNA interference [22], and global DNA demethylation [30]. It looks like a large number of cellular functions have evolved in parallel that make use of the same highly conserved Elongator complex albeit in very different contexts. It remains to be seen which functions of Elongator require its acetylase activity and what would be the corresponding substrates. We are still far from having a comprehensive view of all Elongator functions.

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Review Article

Histone Acetyltransferases as Regulators of Nonhistone Proteins: The Role of Interferon Regulatory Factor Acetylation on Gene Transcription

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When studying transcription factors, it is necessary to investigate posttranslational modifications. Histone acetyltransferases (HATs) are typical of the modification enzymes involved in chromatin regulation. HATs acetylate the transcription factors (nonhistone proteins) as well as histones. Interferon regulatory factors (IRFs) are transcription factors that bind to the interferon regulatory element (IRF-E) and are involved in regulating cell growth, differentiation, and the immune and hematopoietic systems. During the process of binding to a specific DNA element, IRFs also bind to coactivators such as HATs and become modified. This review looks at how IRFs associate with HATs, p300, and PCAF, and thereby contribute to transcriptional activation.

1. Introduction

Cellular proteins are posttranslationally modified by various mechanisms, including acetylation, deacetylation, phosphorylation, and methylation. The reversible acetylation of histone and nonhistone proteins plays a key role in maintaining cellular homeostasis [1, 2]. The acetylation of histones is a prerequisite for transcriptional activation. Transcription factors are recruited to the chromatin by acetylated histones, thereby leading to transcriptional activation. In addition to transcription, the status of histone acetylation may influence cell growth and differentiation [3]. Similarly, the acetylation of several transcription factors may regulate their function, by multiple mechanisms, including the modification of DNA binding ability, secondary protein-protein interactions, protein half-life, and protein localization. These events, in turn, can influence DNA repair, cell cycle progression, apoptosis, and various signaling pathways. Many different transcription factors that are regulated through acetylation have been reported over the past decade.

Interferon regulatory factors (IRFs) were characterized as interferon- (IFN-) responsive transcription factors and were investigated through immune response to pathogens,

immunomodulation, and hematopoietic development [4, 5]. As we will describe below, IRFs are transcription factors that have been shown to be acetylated by histone acetyltransferases (HATs) [6, 7]. Such IRFs modifications are associated with cell growth and differentiation as well as the IFN response. IRFs also associate with several HATs to regulate the transcription of specific genes. Here, we introduce a role for acetylated IRFs or IRF-HAT complexes in specific signaling pathways and cell functions.

2. Histone Acetyltransferases (HATs)

There is a growing body of evidence supporting the notion that acetylation, like phosphorylation, is an important regulatory protein modification. HATs are increasingly being recognized as modifiers of both histones and nonhistone proteins [2]. Many HATs have been identified such as the GNAT superfamily (PCAF, GCN5), p300/CBP, MYST family proteins including MOZ, and nuclear receptor coactivators. HATs function enzymatically by transferring an acetyl group from acetyl-coenzyme A (acetyl-CoA) to an ϵ -amino group of certain lysine side chains within a histone's basic N-terminal tail region. Lysine acetylation neutralizes

part of a histone tail region's positive charge, resulting in weakened histone-DNA or nucleosome-nucleosome interactions [8]. Acetylated chromatin prefers to associate with transcription factors. HATs also acetylate lysine residues within transcription-related proteins as well as histones. HAT proteins form multiple complexes and are recruited to chromatin to acetylate histones or to transcription factors. In terms of IRF regulation by acetylation, PCAF and CBP/p300 play especially important roles.

3. The IRF Family of Transcription Factors

Interferon regulatory factors (IRFs) are transcription factors which participate in the early host responses to pathogens, immunomodulation, and hematopoiesis [5, 9]. Nine mammalian members of the IRF family (IRF-1, -2, -3, -4, -5, -6, -7, -8, and -9) have been identified. All IRFs carry a conserved DNA-binding domain (DBD) consisting of ~110 amino acids in the N-terminal region. The DBD contains a unique helix-turn-helix motif, and it is responsible for binding to the IFN-regulatory factor element (IRF-E) present in the IFN- β promoter [10, 11]. Notably, the consensus sequence of the promoter, G(A)AAAG/CT/CGAAAG/CT/C, is almost indistinguishable from the interferon-stimulated response element (ISRE) activated by IFN signaling. IRF function directly correlates with its sequence-specific binding to the promoters of its regulatory genes. Various post-translational modifications of IRF proteins affect a host's response to pathogens as well as hematopoietic differentiation, immunomodulation, and oncogenesis.

Among the members of the IRF family, IRF-1 and IRF-2 were originally identified through transcriptional studies of the human IFN- β gene [10, 11]. Both IRF-1 and IRF-2 expressed in most cell types and are type I IFN and type II IFN inducible protein. Although IRF-1 and IRF-2 have a high ability of binding to IRF-E, they have opposing activities. Whereas IRF-1 activates transcription from promoters carrying the ISRE, IRF-2 represses the transcription of these promoters in IFN system. Both IRF-1 and IRF-2 mRNAs are expressed at low constitutive levels in the cell, but the IRF-2 protein is more stable and thus accumulates at higher levels (the half-lives of IRF-1 and IRF-2 are 30 min versus 8 h, resp.) [12]. In addition, IRF-1 also acts as a tumor suppressor, and IRF-2 induces cellular transformation [12].

4. Interaction of Histone Acetyltransferases (HATs) with IRF-1 and IRF-2

We previously demonstrated that IRF-1 and IRF-2 both interact with the histone acetyltransferase PCAF in vitro and that this interaction plays an important role in controlling transcription from relevant promoters [6, 13, 14]. IRF-1 stimulates IFN- β promoter with PCAF whereas PCAF enhances IRF-2-dependent H4 promoter activation. Thus, PCAF binding to the IRFs enhances the IRFs-activated promoter. We also demonstrated that the IRF-2 DNA-binding domain interacts with the PCAF bromodomain

[13]. Bromodomain is identified by sequence alignment as a ~60-amino acid motif conserved among *Drosophila Brahma* and female-sterile homeotic (*fsh*) genes and four other potential transcription regulators [15]. Among HATs, PCAF, GCN5, p300, and CBP are bromodomain-containing proteins. The bromodomain of PCAF has a specific affinity for acetyllysine-containing motifs [16]. PCAF bromodomain may associate with the acetylated lysine residue (Lys-75 and Lys-78) within the IRF-2 DNA-binding domain [14]. Although both IRF-1 and IRF-2 are acetylated in vitro [6], the level of acetylation is significantly higher for IRF-2 than for IRF-1. IRF-2 is described as a transcriptional repressor of IFN-responsive genes, and it appears to function by competing with the transcriptional activator IRF-1. We have demonstrated that acetylated IRF-2 inhibits p300-mediated acetylation of core histones [6]. Phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) activate protein kinase C and stimulate the differentiation of U937 cells toward macrophage-like cells. Under these conditions, TPA treatment induces the acetylation of nuclear core histones. IRF-2 associates with p300 and PCAF and becomes acetylated in TPA-treated U937 cells. Acetylated IRF-2 reduces the acetylation of histones H2A and H2B in TPA-stimulated U937 cells. One interpretation of this observed inhibition is that IRF-2, by acting as a substrate for histone acetylases, competitively inhibits histone acetylation (Figure 1(a)). IRF-2 inhibition of histone acetylation is relevant to IRF-2's transcriptional repression of IFN-responsive genes. Another IRF member, v-IRF (derived from Kaposi's sarcoma-associated herpes virus) interacts with p300 to similarly inhibit core histone acetylation, resulting in the repressed transcription of interferon-responsive genes [17].

In contrast, IRF-2 can act as a positive regulator for ISRE-like sequences such as the H4 promoter [18, 19]. IRF-2 is acetylated during the active growth of NIH3T3 cells (i.e., nonconfluent conditions), and acetylated IRF-2 binds to the H4 promoter, thus participating in gene regulation for controlling cell growth. Acetylated IRF-2 may also interact with other proteins, resulting in more efficient gene transcription. Nucleolin, a nuclear protein, preferentially associates with acetylated IRF-2, over nonacetylated IRF-2, resulting in a more efficient transcription of the H4 gene [20] (Figure 1(b)).

IRF-2 binds to PCAF (and p300) and is then subject to acetylation at the lysine residue. Two lysine residues in the DBD, Lys-75, and Lys-78 are found to be the major acetylation sites of IRF-2. Lys-75 is the major site of acetylation by p300 and PCAF whereas Lys-78 is acetylated to a lesser degree. Lys-78 in IRF-2 is a residue conserved throughout members of the IRF family. Amino acid mutation of Lys-78 in IRF-2 led to the abrogation of DNA binding activity independently of acetylation [14]. According to the crystal structure of the IRF-1 DNA-binding domain bound to a DNA target sequence, lysine 78 (corresponding to lysine 92 in IRF-7) stabilizes the protein-DNA complex through hydrogen bonding. Neutralization of the positive charge of this lysine residue effectively tightens the bonds between the transcriptional complex and the promoter [21]. Acetylation of lysine residue of IRF-2 does not alter its DNA binding

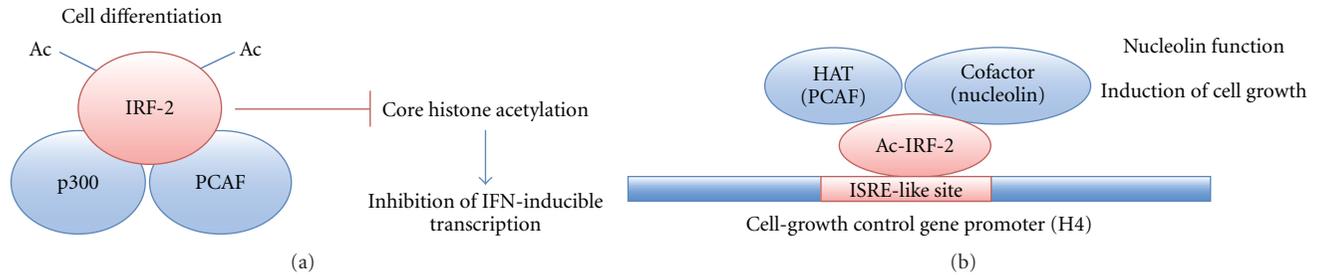


FIGURE 1: Two different functions of interferon regulatory factor-2 (IRF-2) through its acetylation by HAT. (a) During TPA-induced cell differentiation, IRF-2 binds histone acetylases, p300, and PCAF and inhibits core histone acetylation, resulting in the inhibition of IFN-inducible genes. (b) IRF-2 binds to PCAF and is acetylated. Acetylated IRF-2 is recruited to nuclear protein, nucleolin. The IRF-2/PCAF/nucleolin complex contributes to cell growth through the activation of ISRE-like site in the H4 gene promoter.

ability. In the case of IRF-2, acetylation recruits cofactor (nucleolin) to the transcription factor-DNA complex [20].

As mentioned above, IRF-1 is a positive regulator for the IFN-producing system, and stimulates IFN- β promoter with PCAF in the *in vitro* luciferase assay in cultured cells [13]. Dornan et al. reported that the interaction with IRF-1 and p300 is important for synergizing activity of the p53-induced p21 gene activation, independent of IRF-1's DNA binding activity [22]. In their investigation, deletion of the p300-binding sites in IRF-1 eliminates the ability of IRF-1 to stimulate p53 acetylation and associated p53 activity. The nonacetylatable p53 mutant (p53-6KR) cannot be stimulated by IRF-1. They concluded that IRF-1 binding to p300 stabilizes p300 binding to the transactivation domain of p53, including p53 acetylation. Another report indicates that IRF-1 is acetylated and associates with HATs to form a complex that assembles on the HIV-1LTR promoter, and CBP is recruited by IRF-1 to HIV-1LTR promoter even in the absence of Tat [23].

5. IRF-3 and IRF-7

IRF-3 and IRF-7 are involved in IFN gene expression during viral infections. IRF-3 specifically targets IFN- α and IFN- β whereas IRF-7 is required for the induction of additional members of the IFN- α multigene family. In its inactive form, IRF-3 is restricted to the cytoplasm in unstimulated cells. Viral infection or treatment with dsRNA triggers a signal, which results in the specific phosphorylation of specific serine residues in IRF-3. The phosphorylated IRF-3 assembles into a homodimer and then forms a complex with the coactivators CBP/p300 in the nucleus [24, 25]. Specific phosphorylation sites such as Ser-386, were identified for IRF-3 dimerization (activation) [26, 27]. The histone acetyltransferase p300 is required for the DNA binding by the IRF-3 holocomplex, which leads to IFN- β transcriptional activation.

IRF-7 is activated by virus-induced phosphorylation on serine residues within the C-terminal regulatory domain, resulting in induction of the IFN- α gene [28]. Caillaud et al. found that IRF-7 is acetylated by PCAF and GCN5 at a single lysine residue (Lys-92) which is located in the DBD and is conserved throughout the entire IRF family [7]. They reported that acetylated IRF-7 displays an impaired

DNA binding capability and that PCAF repression leads to decreased IRF-7 activity. One may conclude that acetylation of lys-92, negatively modulates IRF-7 DNA binding. As a general rule, if the acetylation occurs within a DNA-binding domain, it will repress the DNA binding, and if it occurs adjacent to a DNA-binding domain, then it will activate DNA binding. Not surprisingly, the precise effect of lysine acetylation depends on the location of the target residue. DNA binding ability is regulated by acetylation of target sites (amino acids) in the case of IRF-7 [7].

Yang et al. reported that IRF-3 and IRF-7 synergistically activate the virus-induced IFN- β promoter in the presence of the p300/CBP coactivator [29]. The IFN- β promoter contains a binding site for the ATF-2/c-Jun, IRFs, and Rel families, and ATF-2, c-Jun, IRF-3, IRF-7, and NF- κ B are associated with the IFN- β promoter in virus-infected cells *in vivo* [30]. The effects of ATF-2/c-Jun, IRF-1, IRF-3/IRF-7, and NF- κ B, on the transcription of -100IFN β CAT reporter in the presence or absence of mammalian p300/CBP have been studied. IRF-1 was able to activate -100IFN β CAT on its own, however, IRF-1 did not stimulate transcription when combined with other factors, more than the sum of their individual effects, regardless of whether or not p300/CBP was present. In contrast, IRF-3/IRF-7 with ATF-2/c-Jun, or NF- κ B showed a synergistic effect in the presence of p300/CBP although only IRF-3/IRF-7 had no effect on the promoter [29]. Moreover, no synergy was observed in the absence of either p300/CBP or IRF-3/IRF-7. p300/CBP play an important role for the functional activation of both IRF-3 and IRF-7 upon virus infection.

6. HATs in Interferon Signaling

The transcription of the IFN- β gene in response to a viral infection requires the assembly of an enhanceosome, consisting of the transcriptional activators NF- κ B, IRF-1, ATF2/c-Jun and HMGI(Y) [31, 32]. The enhanceosome activates transcription by recruiting the CBP/p300 coactivator that is associated with the Pol II holoenzyme complex. CBP and PCAF can acetylate HMGI(Y) at distinct lysine residues, causing opposing effects on IFN- β gene expression. Acetylation of HMGI(Y) by CBP causes enhanceosome disruption leading to the termination of IFN- β gene activation.

Furthermore, CBP-enhanceosome interactions are responsible for the fast recruitment of the PolII holoenzyme, ensuring rapid activation of IFN- β gene expression in response to viral infection [33]. In addition, viral infection induces recruitment of IRF-2 to some of endogenous IFN- β enhancers as part of the enhanceosome assembly process, and IRF-2 incorporation into enhanceosomes restricts the number of IFN- β promoters directing transcription [34]. The repression mechanism of IRF-2 against the enhanceosome has also been investigated. IRF-2 repressed transcription neither by competitive DNA binding nor by directly or indirectly inhibiting assembly of the basal transcriptional machinery directly or indirectly. Senger et al. demonstrated that incorporation of IRF-2 into IFN- β enhanceosomes blocks recruitment of CBP and the associated RNA Pol II and strongly inhibits activation of transcription [34]. This inhibition is not mediated by another protein recruited by IRF-2, but rather by the IRF-2 repression domain itself, while the IRF-2 repression domain interacts with neither the activators nor CBP, but blocks recruitment of CBP.

In contrast, the requirement for deacetylase activity in IFN- α -inducible gene regulation has been reported [35]. IFN- α stimulation induces local histone H4 deacetylation, and the deacetylase HDAC1 associates with both STAT1 and STAT2. The deacetylase activity of HDAC1 acts as a positive coactivator for ISGF3-dependent transcriptional responses and enhances IFN- α -induced transcription. The requirement for deacetylase activity is shared by IFN- γ signaling through STAT1. The role of HDAC1 in IFN responses may reflect an independent activity of HDAC-containing complexes that are associated with transcriptional repression [35].

7. HDAC and IRF

Histone deacetylases (HDACs) are also associated with IRF-dependent transcription. For instance, the HPV E7 protein binds to HDAC and interferes with the transactivation function of IRF-1 by recruiting HDAC to the IFN- β promoter [36]. HPV E7 interferes with the IFN-signaling function of IRF-1 by recruiting HDAC to the specific promoter. IRF-5, another IRF family member, is known to regulate proinflammatory cytokine expression [37]. Feng et al. showed that both HDACs and HATs associate with IRF-5 and alter in its transcriptional activity [38]. They also demonstrated by using trichostatin A (TSA) that ISRE, IFNA, and IL-6 promoters, but not TNF- α , require HDAC activity for transactivation. The HATs CBP and p300 bind and acetylate IRF-5. HDAC and HATs are recruited to IRF-5 in a distinct region and play an important role for transcriptional induction of proinflammatory cytokines mediated by IRF-5.

8. Another IRF Family, c-Myb, and Other Transcription Factors

The IRF family shares homology with the myb oncoproteins. The *c-myb* proto-oncogene product (*c-Myb*) is a member of the helix-turn-helix transcriptional activator family. *c-Myb* regulates differentiation and proliferation in

immature hematopoietic and lymphoid cells, although the relationship of the *c-Myb* family to the interferon system remains undefined [39]. CBP binds via its KIX domain to the activation domain of *c-Myb* and mediates *c-Myb*-dependent transcriptional activation [40]. *c-Myb* interacts with CBP, and the acetylation of *c-Myb* enhances both its affinity for CBP and its capacity for transactivation.

The current list of acetylated proteins includes, amongst others, p53, Myb, GATA-1, Sp1, and MyoD [2, 41, 42]. In most cases, this modification potentiates transcription. Acetylation causes enhanced sequence-specific DNA binding for transcription factors like p53, E2F, EFLF, p50, and PC4 whereas it reduces DNA binding for other factors like Foxo1, HMG1(Y), and p65. The impact upon DNA binding depends on the sites of acetylation. p53 is a sequence-specific transcription factor that is acetylated by p300/CBP [43], and acetylation at lysine residues in the C-terminus of p53 activates its DNA binding. Its function directly depends upon its ability to bind to the promoters of its regulatory genes in a sequence-specific fashion and thus maintain cellular homeostasis. NF- κ B is involved in IFN signaling. The NF- κ B/REL family of transcription factors pivotally controls the inflammatory and immune responses, as well as other genetic programs that are central to cell growth and survival. NF- κ B acetylated by p300/CBP inhibits its interaction with I κ B and induces translocation of the factor to the nucleus. Acetylation of NF- κ B is regulated by the prior phosphorylation. HDAC3 deacetylates NF- κ B, and enables it to bind I κ B and causes its translocation into the cytoplasm. Thus, acetylation, like phosphorylation, is important for regulating the nuclear functions of NF- κ B [44].

9. Future Direction

There are many reports about the hematopoietic and immune regulation of IRFs, which have been demonstrated using primary cells from mice [45–48]. However, it is unclear how acetylation or HAT association with IRFs function affect the immune and hematopoietic systems in vivo. In an IFN-regulating or cell growth control system in vitro, HATs are important factors for the correct functioning of IRFs as well as NF- κ B and p53. IRFs/HATs complex may associate or correlate with other factors including NF- κ B and p53. Recently, other posttranscriptional modification such as methylation, ubiquitination, and sumoylation have also been investigated for many transcription factors. Some of these modifications' targets are also lysine residues. A recent report showed that sumoylation of IRF-1 or IRF-2 regulates their transcriptional activities [49, 50]. We should investigate how these modifications might crosstalk with each other to regulate the transcription factor-mediated homeostasis. Further studies are necessary for clarifying the interaction with acetylation and other modifications of transcription factors.

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Review Article

Androgen Receptor Signalling in Prostate Cancer: The Functional Consequences of Acetylation

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The androgen receptor (AR) is a ligand activated transcription factor and member of the steroid hormone receptor (SHR) subfamily of nuclear receptors. In the early stages of prostate carcinogenesis, tumour growth is dependent on androgens, and AR directly mediates these effects by modulating gene expression. During transcriptional regulation, the AR recruits numerous cofactors with acetylation-modifying enzymatic activity, the best studied include p300/CBP and the p160/SRC family of coactivators. It is known that recruitment of histone acetyltransferases (HATs) and histone deacetylases (HDACs) is key in fine-tuning responses to androgens and is thus likely to play a role in prostate cancer progression. Further, these proteins can also modify the AR itself. The functional consequences of AR acetylation, the role of modifying enzymes in relation to AR transcriptional response, and prostate cancer will be discussed.

1. Introduction

The androgen receptor (AR), a ligand-activated transcription factor and member of the steroid hormone subfamily of the nuclear receptor (NR) superfamily, mediates androgen signalling in the cell. The AR is the only NR that is coded on the X-chromosome, thus males carrying a disease-associated mutation in the gene will be hemizygous and express the disease phenotype. The most prevalent AR-associated disease is prostate cancer, the fifth most commonly diagnosed cancer in the world and second most common cancer among men [1]. The prostate is a gland situated below the bladder, surrounding the urethra. The main function of the prostate is the secretion of components of the seminal fluid, hence, it plays a role in male fertility [2]. Prostate growth is dependent upon androgens, primarily testosterone and its more potent and physiologically active metabolite dihydrotestosterone [3, 4].

The AR is around 919 amino acids long and has distinct structural and functional domains [3, 5] (Figure 1). The N-terminal domain of the receptor is highly flexible, with minimal secondary structure that upon protein-protein

interactions can become more structured [6]. The central DNA-binding domain (DBD) is arranged into three alpha helices organised into two separate zinc finger-like motifs co-ordinated by eight cysteine residues. These helices are important in the recognition of specific DNA sequences, termed androgen response elements (AREs), and DNA-dependent dimerisation of the receptor [3, 7]. The ligand-binding domain (LBD) is situated in the AR C-terminal domain, is important in recognition and docking of androgens, and has been characterised by crystallography [8, 9]. Two transactivation domains exist in the AR: activation function-1 (AF1), situated in the N-terminal domain, and AF2, which is located in the LBD. Unlike other SHRs, and the majority of NRs, the main transactivation potential lies not within the LBD but within the N-terminal domain of the AR [6, 10–12].

The molecular events leading to AR-regulated transcription are outlined in Figure 2. In the absence of androgens, the AR is located in the cytoplasm, in a complex with heat-shock and heat-shock-related proteins. Upon diffusion of androgen into the cell, the globular C-terminal domain of the receptor accepts ligand, and subsequent structural

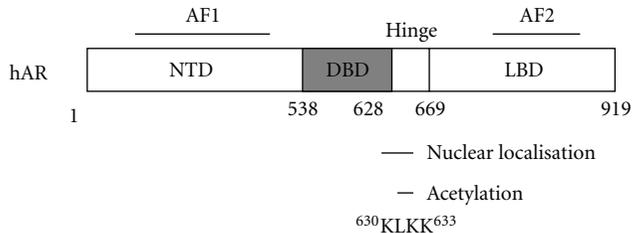


FIGURE 1: Schematic representation of the human androgen receptor (AR). The N-terminal domain (NTD) contains activation function-1 (AF1), which is the major region important in transcriptional activation. The central DNA-binding domain (DBD) is coordinated by two zinc finger motifs and recognises specific androgen response elements. The ligand-binding domain (LBD), situated in the C-terminus, is structurally well characterised and contains the ligand-dependent transactivation domain, AF2. The flexible hinge region connects the structured DBD and LBD and contains both a nuclear localisation sequence and acetylation motif.

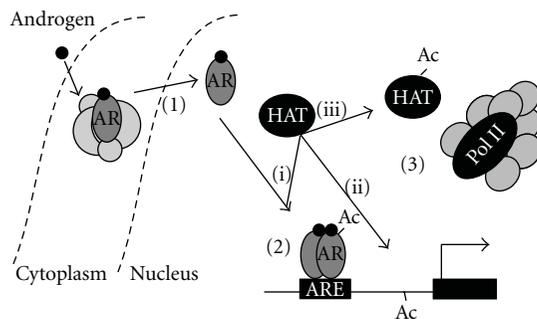


FIGURE 2: Role of acetylation in AR-regulated transcription. In general terms, ligand-dependent transcriptional activation by the AR can be described as follows: (1) ligand-bound AR dissociates from cytoplasmic heat-shock complexes and enters the nucleus. (2) AR binds to specific DNA sequences termed androgen response elements (AREs) and recruits members of the basal transcriptional apparatus. (3) AR recruits a variety of coregulators, which may serve to provide “platforms” for additional coregulator recruitment, regulate the architecture of chromatin directly, and ultimately intensify transcription from target genes. HATs function at a variety of stages including (i) direct acetylation of AR, (ii) acetylation of chromatin, and (iii) acetylation of other factors involved in transcriptional regulation. See text for details.

rearrangements result in dissociation of heat-shock proteins and the exposure of a nuclear localisation sequence (NLS) situated in the hinge region [3]. Nuclear AR binds to AREs located throughout the genome and recruits a variety of cofactors including members of the basal transcription complex and proteins with enzymatic activities such as HATs and HDACs. The formation and identities of these diverse multiprotein complexes result in tight transcriptional regulation of a variety of genes involved in prostate growth, maintenance, and differentiation [6, 13, 14].

In the following review, the impact of acetylation on AR signalling will be discussed. First, modulation of AR activity by direct acetylation will be examined followed by the role of coregulator proteins with HAT/HDAC activity in

AR transcriptional complexes. Finally, the role of acetylation in prostate cancer formation/progression and the application of therapies will be discussed.

2. Androgen Receptor Acetylation

The AR polypeptide has a well-defined structure/function organisation. Increased sophistication and application of bioinformatic tools, the ability to produce high-quality recombinant proteins, and generation of modification-specific monoclonal antibodies have aided the identification of multiple AR modifications. It is well known that the AR exists as a phosphoprotein, and its function is tightly regulated by residue-specific modification by a variety of kinases [15], but only relatively recently has a link been established between direct AR acetylation and protein function. In 2000, the Pestell laboratory identified a short motif ($^{630}\text{KLKK}^{633}$) within the hinge region of AR that has characteristics of an acetylation motif (RXKK) and is conserved between mouse, rat, and human AR (Figure 1) [16]. Using ^{14}C -labelled acetate and recombinant GST-AR fragments, they were able to show that AR was acetylated by p300 and p300/cAMP-response element-binding protein-associated factor (P/CAF) *in vitro*, and further immunoprecipitation experiments confirmed that AR was acetylated by these factors *in vivo* [16]. Combining a peptide-based approach with mass spectrometry, the group mapped the region of acetylation to the AR hinge region (amino acids 623–640) and specifically the KLKK motif and showed that mutation of this motif resulted in a drastically attenuated response to hormone. Furthermore, p300 and P/CAF were unable to potentiate mutant AR activity to the same degree as wild-type, suggesting that acetylation is required for maximal activation [16].

Elegant studies by the same laboratory using acetylation-mimic/gain-of-function mutants confirmed that acetylation plays a key role in AR transactivation with functional consequences; such mutants interacted to a greater extent with p300 than wild-type receptor and enhanced prostate cancer cell growth on soft agar and in xenografts [17, 18]. Interestingly, AR acetylation mimics showed reduced interactions with proteins with histone deacetylase activity (HDAC1) and also transcriptional corepressors (NCoR (Nuclear receptor CoRepressor), Smad3). It is known that p300 can act as a molecular scaffold, recruiting additional coactivators that may modulate the transcriptional response. As acetylation-mimic mutants interacted with p300 to a greater extent than wild-type AR, the authors performed additional cotransfection experiments to test a panel of coactivators. Indeed, it was found that these coactivators could activate acetylation mimics to a greater extent, presumably via increased p300/AR interaction [17].

In addition to p300 and P/CAF, a third protein has been identified that directly acetylates AR, termed Tat-interactive protein, 60 kDa (TIP60) [19]. Originally identified in a yeast two-hybrid screen using domain fragments of AR as bait, TIP60 was shown to interact with the AR-LBD [20]. In this assay, no interaction was observed between TIP60 and

AR N-terminal and DNA-binding domains suggesting that TIP60 may be involved in ligand-dependent transactivation of the AR; however, this interaction is only stabilised in the presence of ligand rather than ligand dependent *per se* [20]. Further characterisation indicated that TIP60 coactivated AR in several cell lines, including the prostate cancer cell line LNCaP and interacts with and augments activity of several other SHRs (progesterone receptor, oestrogen receptor α and β , and glucocorticoid receptor) [20, 21]. Interaction with AR is dependent on an LXXLL motif present in the C-terminal domain of TIP60, mutation of which results in abolition of both interaction and coactivation [21]. Previous research had determined that TIP60 contains an HAT domain and acetylates histone proteins H2A, H3, and H4 [22]. Using combined ^3H -acetyl incorporation and immunoprecipitation assays, Gaughan and coworkers were able to show that TIP60, but not TIP60 HAT-domain mutants, directly acetylates AR *in vivo* [19]. Alanine scanning of the AR acetylation motif abolished coactivation by TIP60 in reporter gene assays providing further evidence that AR acetylation by TIP60 is an important regulatory event [19].

Conversely, proteins that can directly deacetylate AR have been reported. Histone deacetylase 1 (HDAC1) has been shown to interact directly with the AR and repress AR activity [19]. It was observed that HDAC1 interacted with a region of the AR encompassing the DBD/LBD. Furthermore, the deacetylase activity of HDAC1 was required for AR repression and although not proven, the authors suggest that direct deacetylation of AR may result in a transcriptional switch during AR-dependent gene expression [19]. In support of this, both HDAC1 and TIP60 were found to occupy the PSA promoter suggesting that the balance between acetylation and deacetylation is crucial in AR gene regulation [19].

More definitively Sirtuin 1 (SIRT1), an NAD-dependent Class III deacetylase, interacts with, deacetylates, and represses AR activity [23]. A combination of protein-protein interaction assays, use of SIRT1 inhibitors, and a catalytic “dead” SIRT1 mutant indicated that SIRT1 could regulate AR activity through direct deacetylation [23]. Interestingly, it was observed in a mammalian-two-hybrid experiment that SIRT1 abrogated p300-enhanced AR N/C-termini interactions suggesting that this event may be disrupted by deacetylation of AR hinge region [23]. Further experiments utilising an AR acetylation-deficient mutant demonstrated that this conferred resistance to SIRT1-dependent repression and, complementing previous research whereby acetylation enhanced prostate cancer cell line growth, it was found that overexpression of SIRT1 led to a decrease in cellular proliferation and colony formation. Strikingly, it was shown that SIRT1 overexpression had no discernable effect on cell lines lacking AR indicating that growth inhibition was directly linked to SIRT1-dependent AR deacetylation [23].

As outlined in Figure 1, the AR acetylation motif is situated in the flexible hinge region, which connects the structurally defined DNA-binding and ligand-binding domains [3]. Recently, it has become clear that this region impinges on a variety of steps in AR signalling including DNA binding, transactivation, and nuclear localisation [24–26]. Interestingly, this region contains the NLS (amino acids

617–635), which encompasses the acetylation motif. This NLS has characteristics of a bipartite NLS such as that found in nucleoplamin, however, it interacts with importin- α in a fashion that is mechanistically reminiscent of a classical or monopartite NLS [27]. Mutations identified in this region in patients with prostate cancer and androgen insensitivity syndrome (AIS) were examined and shown to confer a ~10–30-fold lower affinity for importin- α compared to wild-type AR. When examined by confocal microscopy, the vast majority of mutant AR proteins translocated more slowly from cytoplasm to nucleus in the presence of ligand [27]. Of direct interest here is a lysine to threonine substitution at amino acid 630 (K630T), found in a patient with prostate cancer, which changes the AR acetylation motif KLKK to TLKK [28]. A combination of confocal microscopy, isothermal calorimetry, and reporter assays indicated that AR_{K630T} had ~30-fold reduced affinity for importin- α ($K_D = 140\ \mu\text{M}$), migrated more slowly into the nucleus upon hormone stimulation, but paradoxically was more transcriptionally active than AR_{WT}[27]. Independently, Fu and coworkers tested this mutant and found that AR_{K630T0020} was resistant to SIRT1-dependent repression [23]. These studies suggest a link between acetylation, nuclear localisation, and prostate cancer, but specific experiments to test this have not yet been reported.

Recently, the concept of interdependent transcription factor modifications has become widespread, particularly in the context of p53 signalling [29], raising the question of interdependency of acetylation and other posttranslational modifications in AR signalling. On this topic, Pestell and colleagues investigated the role of several kinases (AKT, MAPKK, PKA) in signalling through AR wild-type and AR acetylation mutants. It was observed that MAPKK signalling through AR mutant proteins was unaltered, but these mutants did affect both cAMP and AKT signalling [30]. In support of this, inhibition of cAMP signalling led to an increase of wild-type AR, but not an AR acetylation mutant, on the PSA promoter. In addition, well-characterised AR phosphorylation sites were targeted by site-directed mutagenesis to investigate any effect these phosphomutants may have on AR acetylation. Indeed, one phosphorylation site mutant (S94A) showed a decrease in transactivation of a reporter gene in response to the HDAC inhibitor trichostatin A (TSA) relative to wild-type AR [30]. Furthermore, of the three main AR phosphoisoforms, acetylation mutants lacked the hyperphosphorylated 114 kDa form, supporting the hypothesis that acetylation and phosphorylation of AR are interdependent events [30]. Additional experiments must be performed to further characterise the interdependency of AR posttranslational modifications and the functional significance to signalling and gene expression.

3. Androgen Receptor Cofactors with Histone-Acetylation Modifying Activity

Exhaustive lists of AR coactivators, corepressors, and coregulators have been published previously [6, 13, 14, 31]. This section will focus on cofactors with histone-acetylation

modifying activities that have been implicated in AR transcriptional regulation.

The application of chromatin immunoprecipitation (ChIP) to the study of protein-DNA interactions, epigenetics, and transcriptional regulation has vastly increased our knowledge of these processes. Indeed, recent advances in ChIP-chip and ChIP-seq technologies have enabled genome-wide mapping of binding sites for nuclear receptors, including AR [32, 33]. Coregulators with HAT/HDAC activity that have been characterised by ChIP to regulate AR-regulated transcription include p300/CBP, the p160/SRC family, P/CAF, TIP60, and HDAC1 [13].

The best-characterised AR coactivators are p300/CBP and the p160/SRC proteins. These proteins augment the transcriptional activity of multiple NRs and have been shown to be overexpressed in prostate cancer [34–36]. Steroid Receptor Coactivator-1 (SRC1) was identified as an SHR coactivator by the O'Malley laboratory, and sequence and domain interrogation studies led to the discovery that SRC1 contains an HAT domain and that this domain is integral to SHR coactivation [37, 38]. In addition, p160 proteins were further characterised as containing specific sites for nuclear receptor interactions, a CBP interaction domain, and individual activation domains [39]. Further experimentation revealed that SRC1 could potentiate AR activity via separate interactions with AR N- and C-termini suggesting that SRC1 binding is an integral step in full transcriptional activation of AR, possibly by recruiting further coactivators such as p300/CBP [40–42]. CBP and the highly similar p300 were initially characterised as proteins with HAT activity and thus able to regulate transcription [43–45]. Similar to p160/SRC proteins, these factors contain a HAT domain, NR binding sites but additionally sites important for interactions with E1A and CREB proteins [44]. Initial characterisation studies also proposed that these factors could recruit or bind to p160/SRC proteins thus amplifying the transcriptional response [42, 44, 46, 47].

More recently, ChIP-based investigations have reinforced the idea that p160/SRC and p300/CBP factors are central to AR transcriptional regulation. Investigating the response of the AR-regulated gene encoding prostate-specific antigen (*PSA*) to androgen, the Brown laboratory confirmed AR binding to three AREs present in the *PSA* promoter; two proximal to the transcriptional start site and one ARE present in an enhancer region ~4 kb upstream [48]. In addition to AR binding, CBP and SRC2 bound to both enhancer and promoter regions in the presence of hormone, in contrast to HDAC1 and HDAC2 and corepressors SMRT and NCoR which were able to bind *PSA* proximal promoter regions only in the presence of the antiandrogen bicalutamide [48]. Quantitative analysis by the laboratories of Jänne and Palvimo confirmed that SRC2 and p300 could interact with these regions and provided evidence that AR could “load” on enhancer elements to a much higher degree than at proximal promoters. Furthermore, it was observed that different loading kinetics were present, both at enhancer and promoter and between factors, implying another level of complexity in gene regulation [49, 50]. Additional studies confirmed the presence of multiple coactivators at gene enhancers

and promoters, and ChIP-3C established that chromosomal “looping” between these regions could occur, functionally linking distinct enhancer and promoter complexes [48, 51, 52].

TIP60, discussed previously as an AR factor acetyltransferase (FAT), has more recently been found to cycle on and off the *PSA* promoter and enhancer regions, suggesting that this protein may regulate AR-dependent transcription at the chromatin level either through direct acetylation of AR or possibly through acetylation of chromatin [53]. Interestingly, by applying ChIP and re-ChIP techniques the authors observed that the histone deacetylase HDAC1 and the ubiquitin ligase MDM2 (murine double-minute 2) occupied the *PSA* promoter upon treatment with androgen, suggesting these factors may bind simultaneously or even as a complex. Furthermore, MDM2 and HDAC1 were found to co-operatively attenuate AR-regulated transcription linking ubiquitylation, acetylation and deacetylation processes. i.e. (murine double-minute 2) should be after the first reference to the protein [53].

Work from our own laboratory has indicated that proteins belonging to the same family of co-repressors may repress AR-target genes by different mechanisms. We have shown that Hairy/enhancer of split with YRPW-like motif (HEY) proteins repress AR-dependent signalling in a variety of cell lines [54, 55] (and paper submitted). Initially discovered in a yeast-two-hybrid screen to interact with SRC1, HEY1 was characterised as an AR corepressor. HEY1 is sensitive to a variety of HDAC inhibitors and treatment with TSA resulted in de-repression of AR activity, suggesting HEY1 repressed AR activity through the recruitment of class I/II HDACs [55]. Similar experiments performed on HEYL, a third member of the HEY family, indicated that class I/II HDAC activity was not required for AR repression (paper submitted), hence HEY proteins may employ a variety of possible mechanisms to repress AR activity.

The AR regulates multiple and varied genes at the level of transcription thus is reliant on the recruitment of distinct coregulator complexes to fine-tune transcriptional responses. It is apparent from numerous studies employing ChIP that AR recruits proteins with both HAT and HDAC activities, such as p160/SRC, p300/CBP, and HDAC1, to achieve the desired control. The emergence of genome-wide ChIP will allow the dissection and comparison of transcriptional complexes at multiple genes and specific patterns of histone modifications, which will further enhance our knowledge of important coregulators.

4. Acetylation and Prostate Cancer

As stated above, direct acetylation of AR in response to androgen stimulation increases AR activity, response element binding, and cellular proliferation [17, 30, 56, 57]. The role of acetylation in AR activity was confirmed using acetylation-mimic mutants, which were found to interact with p300 to a higher degree than wild-type AR. Additionally, a panel of coactivators, such as SRC1 and TIP60, were found to coactivate these mutant ARs more robustly than wild-type

receptor. Furthermore, these mutants conferred a growth advantage to tumour cells *in vitro* and *in vivo* suggesting that AR acetylation may play an important role in the development and/or progression of prostate cancer [16, 17, 56]. These data have been supported by the findings that SIRT1, an NAD-dependent HDAC, can deacetylate AR and reduce cellular proliferation and colony formation [23].

Androgens drive prostate tumour growth hence therapies are directed towards reducing levels of circulating androgens and AR activation via administration of luteinising-hormone releasing hormone (LHRH) analogues and/or anti-androgens. Patients generally respond well to treatment but in the majority of cases tumours progress to the advanced, androgen-independent/hormone-refractory stage of the disease. Several mechanisms can drive progression to androgen-independent prostate cancer including amplification of AR, altered levels of co-factors and somatic AR mutations that result in promiscuous activation of the receptor by non-androgenic ligands and growth factor pathways. [4, 58, 59]. Patients diagnosed with androgen-independent prostate cancer have a median survival time of 12–18 months; thus, it is important to develop new treatment strategies to combat disease transition. One new development is the reduction in levels of circulating adrenal androgens using drugs such as Ketoconazole and Abiraterone, which are p450 inhibitors targeting steroid biosynthesis pathways [58, 60].

Several HDAC inhibitors are currently undergoing testing in clinical trials including suberoylanilide hydroxamic acid (SAHA), LBH589 and Depsipeptide, although results suggest that these treatments have moderate effects [58, 61]. It may seem paradoxical that HDAC inhibitors are used as prostate cancer therapies since acetylation of AR increases cellular proliferation as outlined above. However, it has been proposed that HDAC inhibitors act via HDAC6 thus acetylating HSP90, known to be central in AR folding and ligand binding, or by sensitizing cells to DNA damage [58, 62, 63]. A limited number of HAT inhibitors are in preclinical or clinical trials, with p300/CBP and P/CAF-specific inhibitors showing the most promising effects [64, 65]. Curcumin, a naturally occurring HAT inhibitor, has been shown to inhibit CBP-dependent acetylation of histones and non-histone proteins and seems to have a pro-apoptotic effect on prostate cancer cells *in vitro* and *in vivo* [64, 66, 67].

5. Concluding Remarks

Prostate cancer growth, in early stages, is driven by androgens, and hence AR is central to disease formation and progression. As a transcription factor, AR recruits and is regulated by multiple proteins with a variety of enzymatic functions, including proteins with the ability to modify the acetylation status of chromatin and also the AR itself. These modifications allow AR to directly and acutely regulate the hormonal response, protein-protein interactions, transcriptional complexes, and importantly cellular proliferation. These observations, together with the findings that levels of cofactors with HAT/HDAC activity are overexpressed in

patients with prostate cancer, have resulted in acetylation becoming an important therapeutic target.

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Research Article

HDAC Activity Is Required for Efficient Core Promoter Function at the Mouse Mammary Tumor Virus Promoter

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Histone deacetylases (HDACs) have been shown to be required for basal or inducible transcription at a variety of genes by poorly understood mechanisms. We demonstrated previously that HDAC inhibition rapidly repressed transcription from the mouse mammary tumor virus (MMTV) promoter by a mechanism that does not require the binding of upstream transcription factors. In the current study, we find that HDACs work through the core promoter sequences of MMTV as well as those of several cellular genes to facilitate transcriptional initiation through deacetylation of nonhistone proteins.

1. Introduction

Histone deacetylases (HDACs) comprise a large and varied family of proteins which are divided into four classes based on structural homologies [1]. Interest in their role in development, physiology, and cell biology has been intensified by the development of small molecule inhibitors of class I (HDACs 1–3, 8) and class II (HDACs 4–7, 10) family members as anticancer drugs. Two such inhibitors, Vorinostat and Romidepsin, are now approved for treatment of advanced cutaneous T-cell lymphoma and many more are being evaluated in clinical trials [2]. These drugs are also being considered for use in other diseases including cardiovascular, neurodegenerative, and inflammatory disorders [3–5]. In spite of their current and potential clinical use, the precise roles of HDACs in regulating cellular processes through protein acetylation are largely unknown. HDACs are thought to control cell growth and survival through both transcriptional and nontranscriptional pathways that may be partially specific to cell type (reviewed in [6]). Currently our lack of knowledge about the general and cell-specific functions of HDACs limits our ability to predict whether particular tumors will respond to HDAC inhibitors and

which other therapeutics might work synergistically with these drugs in treating various cancers.

Long-standing models of the role of acetylation in transcription generally cast histone acetyltransferases (HATs) as coactivators and HDACs as corepressors [7]. This is based on the positive relationship between histone acetylation and transcription and the fact that many transcriptional activators recruit HATs to target promoters. In contrast, there is a negative correlation between histone hypoacetylation and transcription, and HDACs are often found in complexes that work to repress transcription. This model, however, is inadequate to explain all the roles of HDACs in transcription. Expression profiling of cells treated with HDAC inhibitors [8–14] shows that genes are both activated and repressed by these drugs. Gene repression is observed even within 2 h of treatment when effects on transcription are likely to be the direct result of HDAC inhibition rather than secondary effects dependent on prior changes in gene expression. In addition, studies of individual genes have demonstrated direct repressive transcriptional effects of HDAC inhibitors or knockdown of particular HDACs (reviewed in [15, 16]). Furthermore, transcriptional activators such as glucocorticoid receptor (GR) and STATs 1, 2, and 5 have been shown

to interact with HDACs at target genes to facilitate transcription [17–20]. Finally, a recent genomewide mapping study showed that in addition to HATs, HDACs are enriched at the promoters of active genes and are recruited along with HATs to transcriptionally inducible genes [21]. Taken together, this accumulated evidence indicates that HDACs serve an important role in the activation of transcription and/or maintenance of the activated state. However, the mechanisms by which HDACs facilitate transcription are poorly understood.

One example of a promoter that is repressed by HDAC inhibitors [22–26] or HDAC knockdown [17] is that of the mouse mammary tumor virus (MMTV), a well-studied model system for glucocorticoid receptor- (GR-) mediated activation of transcription. Initially, it was reported that transcriptional activation of this promoter by glucocorticoids was significantly impaired in the presence of HDAC inhibitors [23]. Later, we and others showed that basal MMTV transcription was also repressed [22, 24]. We demonstrated that this repression was very rapid and potent but occurred independently of changes in histone acetylation, chromatin remodeling, or chromatin structure [22]. Our analysis of the sequences required for repression showed that sequences upstream of the TATA box were dispensable. We concluded that HDACs facilitated MMTV transcription through nonhistone proteins which are essential for efficient basal transcription. More recently, Qiu et al. [17] showed that HDAC1 can be found in GR complexes and is associated with the MMTV promoter in the presence and absence of glucocorticoids. In addition, they showed that siRNA-mediated knockdown of HDAC1 impaired the ability of GR to activate the promoter. Together these data make a strong case that HDAC activity is required to facilitate both basal and activated transcription at the MMTV promoter.

In the current study, we have investigated the mechanism by which HDAC inhibition impairs basal transcription. Key to our findings was the development of an in vitro transcription system that recapitulates repression of MMTV transcription due to inhibition of HDAC activity. Surprisingly we find that the repression is conferred through the MMTV core promoter and involves impaired transcriptional initiation. Our results have revealed a novel role of HDAC activity in promoting transcription.

2. Materials and Methods

2.1. Cell Culture. Cell line 1470.2 is derived from C127i mouse mammary adenocarcinoma cells and contains stably replicating copies of a transcription unit consisting of the MMTV long terminal repeat (LTR) driving expression of the chloramphenicol acetyltransferase (CAT) gene. Both 1470.2 and HeLa cells were maintained in Dulbecco's Media Essential Medium (DMEM), containing 10% fetal bovine serum.

2.2. Plasmids. pMluc and pMTV(TATA/+100)luc have been previously described [22]. pMTV(TATA-DPE)luc contains MMTV LTR sequences from –40 to +32 bp. pMTV(TATA-Inr)luc contains MMTV LTR sequences from –40 to +4 bp.

Both are cloned into pXP1, a promoterless luciferase reporter construct [27]. The luciferase constructs containing core promoters from cytomegalovirus (CMV), c-fos, interleukin 4 (IL4), and carbonic anhydrase 2 (CA2) were generated by cloning annealed oligonucleotides with sequences from the TATA box to Inr element for each gene into pXP1. pAdML was a gift from Dr. Gordon Hager (NCI, NIH, Bethesda, MD). It contains a 190 bp G-free cassette 3' to the Adenovirus Major Late (AdML) promoter. pMTV-InrA-G, the MMTV template used for in vitro transcription, was constructed by subcloning annealed oligonucleotides containing MMTV LTR sequences (–39 to +5 bp) just 5' of the 400 bp G-free cassette of pC₂AT19 (a gift from Dr. Robert Roeder, Rockefeller University). The transcription start site of the MMTV promoter is a G nucleotide. It was modified to an A in pMTV-InrA-G for use with the G-free cassette. This modification did not affect core promoter activity under any of the conditions tested in this study (data not shown).

2.3. Transfection Assays. DNA was transiently transfected into HeLa cells using the calcium phosphate method according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Two days after transfection, cells were treated and harvested as described previously [22]. Cell line 1470.2 was transfected by electroporation as described [22]. Treatment and harvest was carried out 1 day after transfection. Lysates from both cell lines were assayed for protein concentration and luciferase activity. Luciferase activities were normalized to protein concentration for each sample.

2.4. Chromatin Immunoprecipitation (ChIP) Assay. The ChIP assay was performed as described by Magklara and Smith [28] with slight modification. Briefly, 1470.2 cells were grown to 95% confluency on 150 mm plates and treated with 50 ng/ml TSA or 100 nM dexamethasone (Dex) for 15, 30, or 60 minutes. The cells were fixed with 1% formaldehyde for 10 minutes at 37°C and neutralized with 0.125 M glycine. After collection of cells by scraping, they were washed 3X with cold PBS and incubated with lysis buffer (1% SDS, 10 mM EDTA, 50 mM TrisHCl, pH8.0) for 10 minutes on ice. After centrifugation cells were resuspended in 1.5 ml lysis buffer and sonicated. Chromatin was precleared with a mixture of protein A and G agarose beads containing salmon sperm DNA. The supernatants were then incubated with 3 to 5 µg 8WG16 RNA polymerase II antibody (Covance) for 16 h at 4°C. Protein A and G agarose beads were added and incubation continued for 2 h. Subsequently, chromatin-bead complexes were washed once each with low salt buffer (0.1% SDS, 1% Triton-X100, 2 mM EDTA, 20 mM Tri-HCl, pH 8.0, 50 mM NaCl), high salt buffer (0.1% SDS, 1% Triton-X100, 2 mM EDTA, 20 mM Tri-HCl, pH 8.0, 500 mM NaCl), and lithium chloride buffer (250 mM LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl) and twice with TE buffer. All buffers contained protease inhibitors. Bound chromatin was eluted successively with 1.5% SDS buffer (1.5% SDS, 0.1 M NaHCO₃) and 0.5% SDS buffer (0.5% SDS, 0.1 M NaHCO₃). Crosslinks were reversed by incubation at 65°C for 5 h in the presence of 200 mM NaCl

and 10 μg RNase A. Protein was digested at 45°C for 2 h in the presence of 10 mM EDTA, 40 mM Tris-HCl pH6.5, and 20 μg proteinase K. DNA was extracted twice with phenol-chloroform-isoamyl alcohol and precipitated with ethanol in the presence of glycogen. Detection of MMTV sequences was carried out by PCR analysis using the following primers: MMTV promoter/5' transcribed region—Forward-5' TTTCCATACCAAGGAGGGGACAGTG 3', Reverse-5' CT-TACTTAAGCCTTGGGAACCGCAA 3', CAT coding region (CDS)—Forward-5' CCGTTTTACCATGGGCAA 3', Reverse-5' AAGCATTCTGCCGACATGGA 3'. In each experiment, cycle number was empirically adjusted so that amplification of the desired sequences was in the linear range. Generally, cycle number varied between 22 and 28 cycles.

2.5. HeLa Nuclear Extract Preparation. HeLa S3 cell pellets were purchased from the National Cell Culture Center. Cultures of HeLa S3 cells (5 liters at 0.5×10^6 cell/ml) were treated with or without TSA (50 ng/ml) for 2 h. Cells were pelleted and flash frozen prior to overnight shipping. Nuclear extracts were prepared by the method of Kusk et al. [29] with modification. All procedures were conducted at 4°C and all buffers used in the extraction procedure contained 500 μM AEBSE, 150 nM aprotinin, 1 μM E-64, 0.5 mM EDTA, and 1 μM leupeptin. TSA (50 ng/ml) was included in all buffers used for generation of nuclear extracts from TSA-treated cells. Upon receiving the cells, they were thawed and washed once with ice-cold PBS and resuspended in five packed cell volumes (PCV) of HB1 (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.75 mM spermidine, and 0.15 mM spermine). After incubation on ice for 10 minutes, the cells were centrifuged and resuspended in two PCV HB1. The cells were subsequently lysed by Dounce homogenization. One-tenth volume of HB2 (67.5% sucrose in HB1) was added, and the homogenates were centrifuged at $16,000 \times g$ for 2 minutes. The nuclear pellets were resuspended in NLB (20 mM HEPES pH 7.6, 100 mM KCl, 12.5 mM MgCl₂, 20% glycerol, 0.2 mM EGTA, 0.2 mM EDTA, and 2 mM DTT) and rotated for 30 minutes. The nuclear lysates were centrifuged ($150,000 \times g$, 90 minutes), and the pellets were mixed with 0.33 g solid ammonium sulfate/ml and resuspended in NLB. After rotation for 20 minutes, precipitated proteins were collected by centrifugation ($85,000 \times g$, 20 minutes) and resuspended gradually in approximately 1 ml dialysis buffer (20 mM HEPES pH 7.6, 100 mM KCl, 12.5 mM MgCl₂, 20% glycerol, 0.2 mM EGTA, 0.2 mM EDTA, and 2 mM DTT). The extracts were dialyzed twice for 2 h in 1 liter of dialysis buffer. Supernatant was collected after centrifugation ($18,000 \times g$, 20 minutes), aliquoted, and stored at -80°C.

2.6. In Vitro Transcription Assay. The assay was performed as described by Kusk et al. [29] with minor modification. The ratio of nuclear extract protein to DNA was optimized for each set of nuclear extracts. In general, approximately 60 μg extract protein was used with 1200–1500 ng DNA template per reaction. Briefly, nuclear extract was incubated at 30°C for 60 minutes with DNA template in a 30 μl reaction mixture containing 20 mM HEPES, pH7.9, 60 mM

KCl, 2 mM DTT, 5 mM creatine phosphate, 1 mM 3'-O-methylGTP, 0.5 mM ATP, 0.5 mM CTP, 20 μM UTP, 5 mM MgCl₂, 10 μCi [α -³²P]UTP (400 Ci/mmol), 10% glycerol, and 10 U RNase T1. The reaction was terminated by addition of 300 μl of Stop Solution (20 mM EDTA, 0.2 M NaCl, 1% SDS, and 10 μg tRNA). RNA transcripts were extracted twice with phenol/chloroform/isoamyl alcohol followed by ethanol precipitation. The transcripts were resuspended in denaturing loading dye, incubated at 95°C for 3–4 minutes, and separated in 6% denaturing polyacrylamide gels (Sequagel, National Diagnostics). Gels were dried and exposed to phosphorimaging screens. Radiolabeled transcripts were visualized with a Storm phosphorimager (Molecular Dynamics, Sunnyvale, CA).

For competition assays, nuclear extracts were preincubated with different amounts of the pAdML template for 30 minutes at 30°C. Subsequently, the in vitro transcription reaction mixture containing NTPs and the MMTV template was added, and the transcription was allowed to proceed for 30 minutes at 30°C. For analysis of de novo transcription, nuclear extracts were incubated with either MMTV or AdML template for 10 minutes at 30°C after which in vitro reaction mixture was added. Approximately 30 seconds later, water or Sarkosyl (0.05% final concentration) was added, and transcription was allowed to proceed for 30 min before the reaction was stopped and transcript production was analyzed.

2.7. HDAC Assay. HDAC assays were carried out as described [17]. Nuclear extract protein (20 μg) was diluted in assay buffer (20 mM Tris HCl, 150 mM NaCl, 0.5 mM EDTA, and 5% glycerol) in the presence or absence of TSA (50 ng/ml). The mixture was incubated for 30 min at 30°C to allow for HDAC inhibition. Subsequently, ³H-acetylated histones prepared from sodium butyrate-treated HeLa cells were added, and incubation continued for an additional 30 min. The reaction was stopped with denaturing buffer (1.44 M HCl and 0.24 M acetic acid). Tritiated acetyl groups released by HDAC activity were extracted using ethyl acetate. Released cpm's were measured by scintillation counting.

3. Results

3.1. HDAC Activity Is Required for Transcription from TATA Box Containing Core Promoters. Our previous study showed that the repression of MMTV transcription by HDAC inhibitors did not require sequences upstream of the TATA box [22]. Repression was conferred through a region of the promoter that extended from the TATA box to 100 bp downstream of the transcription start site. In the current study, we further dissected the sequences required for repression. In particular, we were interested in determining the role of the core promoter, a region roughly defined as -40 to +40 bp relative to the start site of transcription (TSS). As reviewed in [30], the core promoter of a gene is made up of a series of elements which are not common to every core promoter but are generally found in subsets, depending on the particular gene. These include the upstream and

downstream TFIIB recognition elements (BREs), the TATA box, and the initiator element (Inr), all of which are located upstream of the TSS with the exception of the Inr, which is located around the TSS. Some core promoters also contain elements downstream of the TSS including the downstream promoter element (DPE), the motif ten element (MTE), and the downstream core element (DCE).

The MMTV promoter contains a functional TATA box and initiator [31] but does not have BRE sequences. Downstream of the TSS there is a sequence that has some homology to the DPE consensus. To determine whether any of these elements confer repression mediated by HDAC inhibition, a series of luciferase reporter constructs were generated that contain various fragments of the MMTV promoter as shown in Figure 1(a). These constructs were transfected into HeLa cells or 1470.2 cells, which were derived from the C127i mouse mammary adenocarcinoma cell line as described in Section 2. Consistent with our previous study, repression by TSA is not dependent on cisacting sequences upstream of the TATA box (compare pMluc with pMTV(TATA/+100)luc). Repression is also conferred by a fragment extending from the TATA box to the sequence resembling the DPE [pMTV(TATA-DPE)luc]. However, the DPE-like sequence is not required because a construct containing sequences between the TATA box and Inr [pMTV(TATA-Inr)luc] is sufficient to confer TSA-induced repression in both cell lines. Thus, we conclude that the repression of MMTV transcription mediated by HDAC inhibition is dependent solely on its functional core promoter elements.

In our previous study, we also showed that minimal promoter sequences from other viral and cellular genes were repressed by TSA treatment [22]. It is therefore possible that basal transcription of other core promoters is also sensitive to HDAC inhibition. Reporter constructs containing the analogous region from several viral and cellular TATA box-containing promoters were generated and transfected into HeLa or 1470.2 cells. The results (Figure 2) show that TSA causes repression of core promoters from all genes tested, indicating that transcription dependent on the TATA to Inr region is generally sensitive to HDAC inhibition.

3.2. HDAC Activity Is Required for Transcriptional Initiation at the MMTV Promoter. The sensitivity of the MMTV core promoter to HDAC activity could be due to a role for HDACs in regulation of either transcriptional initiation or elongation. In the case of the former, the recruitment of RNA polymerase II (RNA pol II) may be impaired by HDAC inhibition. In the case of the latter, polymerase recruitment may be unaffected, but its ability to clear the 5' transcribed region (first 100 bp) may be impaired due to pausing or stalling. To distinguish between these possibilities, we performed chromatin immunoprecipitation (ChIP) assays to measure the association of RNA pol II with the core promoter/5' transcribed region. If transcriptional initiation is impaired by HDAC inhibition, polymerase association with the core promoter may decrease due to a defect in polymerase recruitment. If HDAC activity is required to prevent pausing or stalling of polymerase, the association of RNA pol II with

the 5' transcribed region would be unchanged or somewhat increased as stalled polymerase accumulates. In the ChIP assays, we used an antibody that preferentially binds to RNA pol II with a hypophosphorylated C-terminal domain (CTD). This antibody has been shown to recognize both initiating as well as 5' paused polymerase [32, 33] which does not contain a highly phosphorylated CTD because serine 2 phosphorylation has not yet occurred [34].

Cell line 1470.2 contains stably replicating copies of a transcription unit consisting of the full-length MMTV LTR/promoter driving the expression of the chloramphenicol acetyltransferase (CAT) reporter gene. Basal and activated expression of the MMTV-CAT gene in this cell line has been previously shown to be inhibited by TSA treatment [22]. Cells were treated with TSA for various times shown in Figure 3. As a positive control for RNA pol II association with the MMTV promoter, we included chromatin from cells treated with the synthetic glucocorticoid, Dexamethasone (Dex), which induces transcription from the promoter. As expected [35], Dex treatment rapidly but transiently induces recruitment of RNA pol II to the MMTV promoter (Figure 3(a), left panel). There is no association with downstream sequences in the CAT coding region (CAT CDS) that are well separated from the core promoter region, thus confirming that the antibody preferentially recognizes the polymerase in its initiating rather than its hyperphosphorylated elongating form.

In the presence of TSA, the association of RNA pol II with the core promoter/5' transcribed region clearly decreases over a time frame of 60 minutes (Figure 3(a), right panel). A quantitative representation of RNA pol II association data from 2 to 3 experiments with TSA is shown in Figure 3(b). By 60 minutes of TSA treatment, there is a 60% loss of polymerase. This is in good agreement with the magnitude of repression observed in the transfection experiments shown in Figure 1. In addition, both the kinetics and magnitude of TSA-induced decrease in promoter association of RNA pol II are highly consistent with changes in the rate of transcription from the MMTV promoter as measured by nuclear run-on in our previous study, in which we observed a 60–70% drop in transcription from the MMTV-CAT gene within 30–60 minutes of TSA treatment [22]. From these data, we conclude that HDAC activity is required in some way for efficient RNA pol II recruitment and transcriptional initiation.

3.3. Development of an In Vitro Transcription System to Study the Role of HDACs in Transcriptional Initiation. Our previous study showed that HDAC inhibition does not lead to changes in chromatin structure, chromatin remodeling, or large increases in histone acetylation at the MMTV promoter [22], leading us to hypothesize that HDACs play a role in regulating the interactions or functions of nonhistone proteins essential for transcription from this promoter context. Thus, the mechanisms by which HDAC activity impact core promoter activity could be precisely defined by in vitro transcription assays, which allow a level of experimental control not possible in cell-based assays. To develop such an assay, we adopted the methods of Kusk et al.

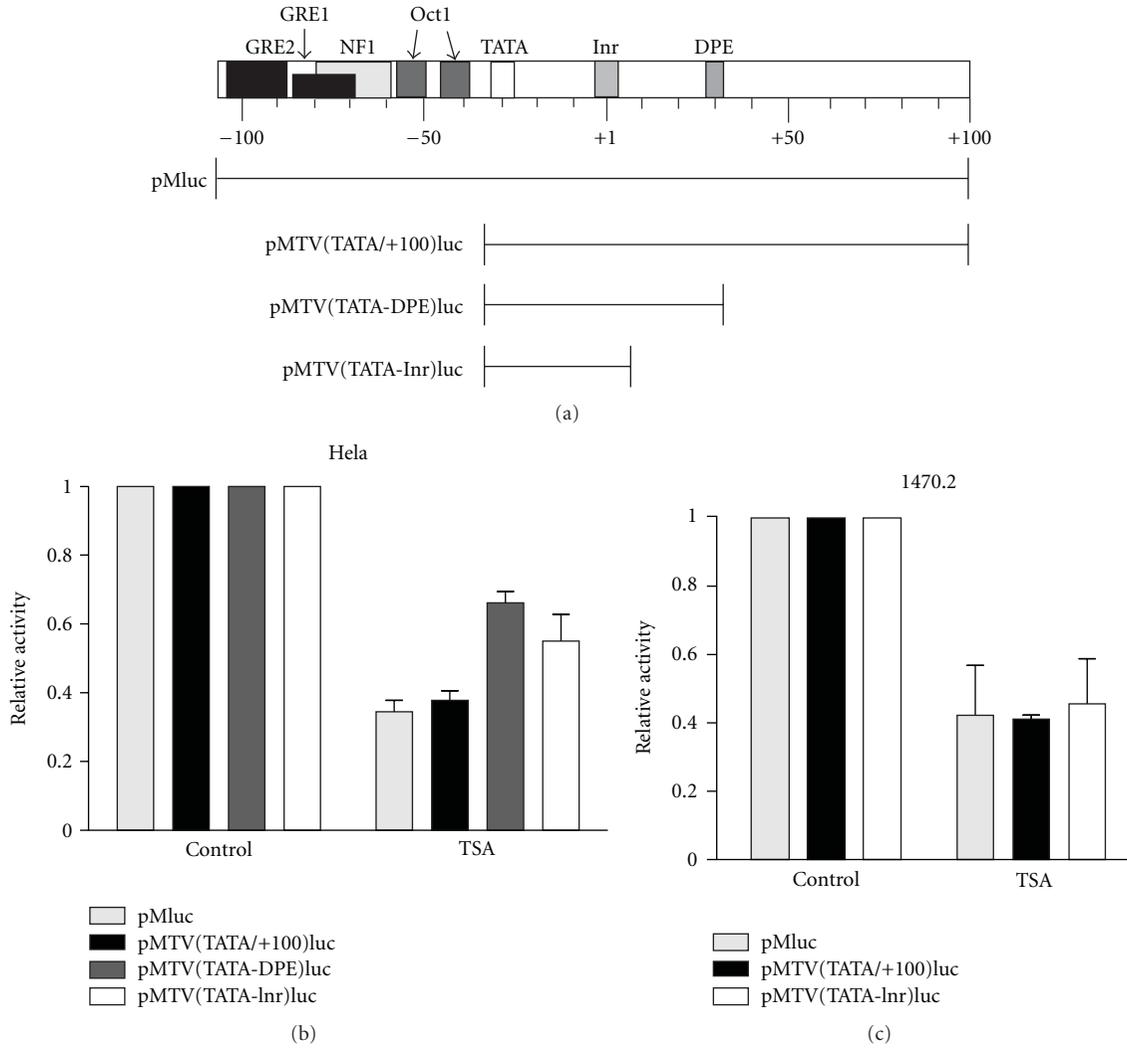


FIGURE 1: The MMTV core promoter confers transcriptional repression by the HDAC inhibitor, TSA. Various luciferase reporter constructs containing fragments of the MMTV promoter shown in (a) were transiently transfected into HeLa or 1470.2 cells and treated with or without TSA (50 ng/ml) for 6 h prior to harvest. Cell lysates were assayed for luciferase activity, and these values were normalized to protein concentration for each sample. Normalized values for untreated (control) samples were set to 1, and values for treated samples were expressed as a fraction. Statistical analysis was performed on results from 3 to 7 independent experiments. Results from HeLa and 1470.2 cells are shown in (b) and (c), respectively. Error bars represent SEM.

[29] who had measured MMTV core promoter function *in vitro* using templates with G-free cassettes [36]. Our goal was to compare transcription in nuclear extracts generated from cells treated with TSA to those generated from untreated cells to determine whether the repression observed in cell-based assays could be recapitulated *in vitro*. Because they can be easily grown in large quantities, we turned to HeLa S3 cells to generate nuclear extracts by the strategy outlined in Figure 4(a). In the case of extracts made from cells treated with TSA, we maintained TSA in all buffers during extract preparation to prevent HDACs from becoming reactivated.

We chose to compare transcription of two promoters: MMTV and adenovirus major late (AdML). We designed an MMTV template containing the core promoter from the TATA box to the Inr upstream of a G-free cassette of

approximately 400 bp (Figure 4(b)). The AdML template contains a larger promoter fragment upstream of a smaller G-free cassette. These templates were transcribed using equal quantities of nuclear extract from control (C) and TSA-treated (T) cells as shown in Figure 4(c). While transcription from the AdML template was similar in the two extracts, MMTV transcription was reduced in the extracts from TSA-treated cells. To eliminate the possibility that these results were due to differences in transcriptional competency specific to a particular set of extracts, we generated two additional sets of extracts (control and TSA-treated) and performed the same experiment. Figure 4(d) shows that the patterns of transcription from the two templates are very reproducible. MMTV transcription was consistently reduced in extracts from TSA-treated cells relative to a

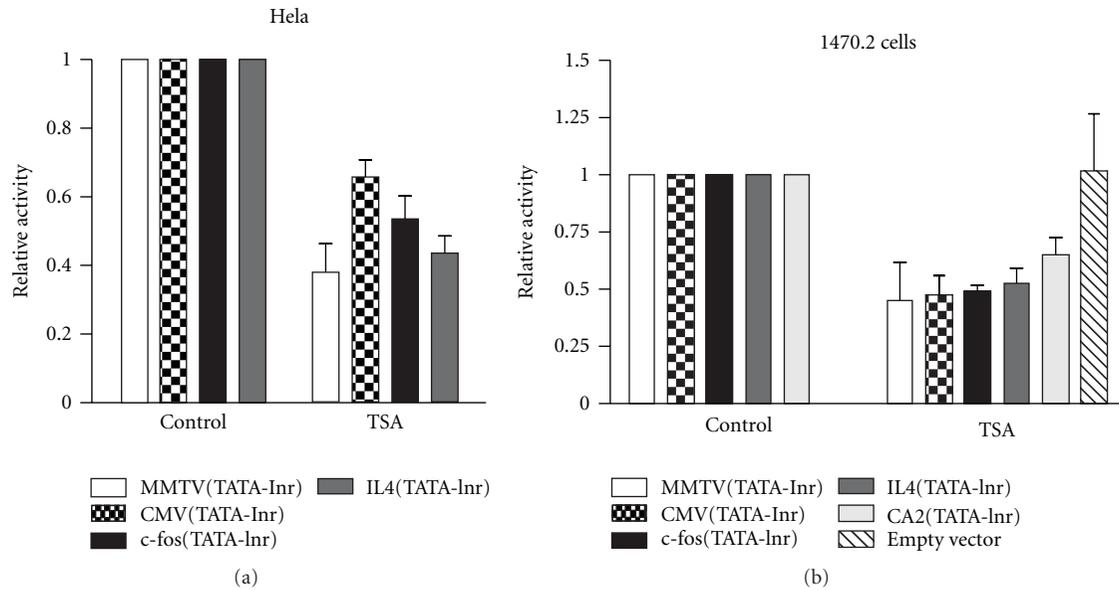


FIGURE 2: Transcription dependent on core promoters is repressed by TSA. Luciferase reporter constructs were generated which contain core promoters (TATA to Inr sequences) from several viral and cellular genes, including Cytomegalovirus (CMV), c-fos, interleukin 4 (IL4), and carbonic anhydrase II (CA2). These constructs were transiently transfected into either HeLa or 1470.2 cells which were treated with or without TSA (50 ng/ml) for 6 h. Cell lysates were assayed for luciferase activity, and the data was processed as described in the legend to Figure 1. Results from at least 3 independent experiments done with HeLa or 1470.2 cells are shown. Error bars represent SEM.

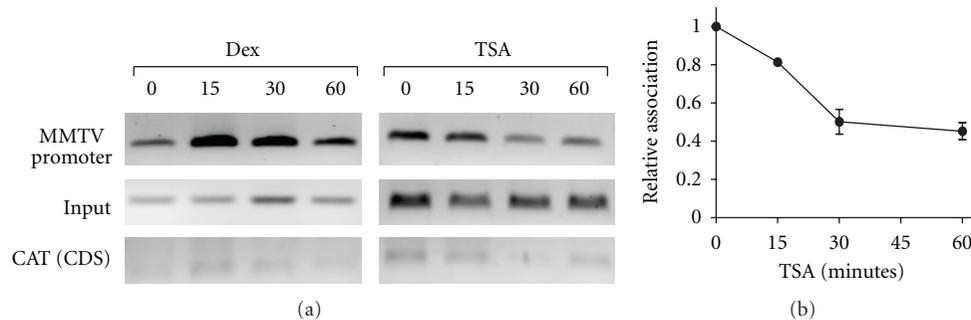


FIGURE 3: Inhibition of HDACs results in the loss of initiating RNA pol II from the MMTV promoter. 1470.2 cells were treated with either Dex (100 nM) or TSA (50 ng/ml) for 0, 15, 30, or 60 minutes, after which cells were processed for ChIP assay. Chromatin was immunoprecipitated with an RNA pol II antibody that recognizes the hypophosphorylated form of the CTD. Conventional PCR was performed on input and immunoprecipitated DNA using primers specific for the MMTV promoter and the coding region (CDS) of the CAT reporter. PCR results from a representative experiment are shown in (a). A graphical representation of the data from 2 to 3 independent experiments is shown in (b) for the TSA time course. Values from control samples were set to 1, and values from TSA-treated samples are expressed as a fraction. Error bars represent SEM.

matched control extract while AdML transcription was unaffected. We therefore conclude that the repression of MMTV transcription we observe in cell-based assays is recapitulated *in vitro*, and that this system can be utilized to ask precise mechanistic questions about the role of HDAC activity in transcriptional initiation.

3.4. Transcriptional Reinitiation Rather Than De Novo Transcription Is Sensitive to HDAC Inhibition. Transcriptional initiation is a broad term that incorporates two distinct processes: de novo transcription, which is the initial assembly of a preinitiation complex (PIC) and the first round of

transcription, and reinitiation, which is the recruitment of RNA pol II to a pre-existing scaffold complex that is distinct in composition from the PIC. De novo transcription can be differentiated from reinitiation with the use of the detergent sarkosyl [37, 38]. If added to a preassembled PIC prior to the addition of nucleotide triphosphates (NTPs), sarkosyl (0.05%) will inhibit all transcription as shown for the AdML template in Figure 5(a). However, if it is added several seconds after NTP addition, the initial round of transcription will proceed and reinitiation is inhibited. As seen in Figure 5(a), this results in a significantly lower amount of transcript because only de novo transcription occurs. To determine whether HDAC activity is involved

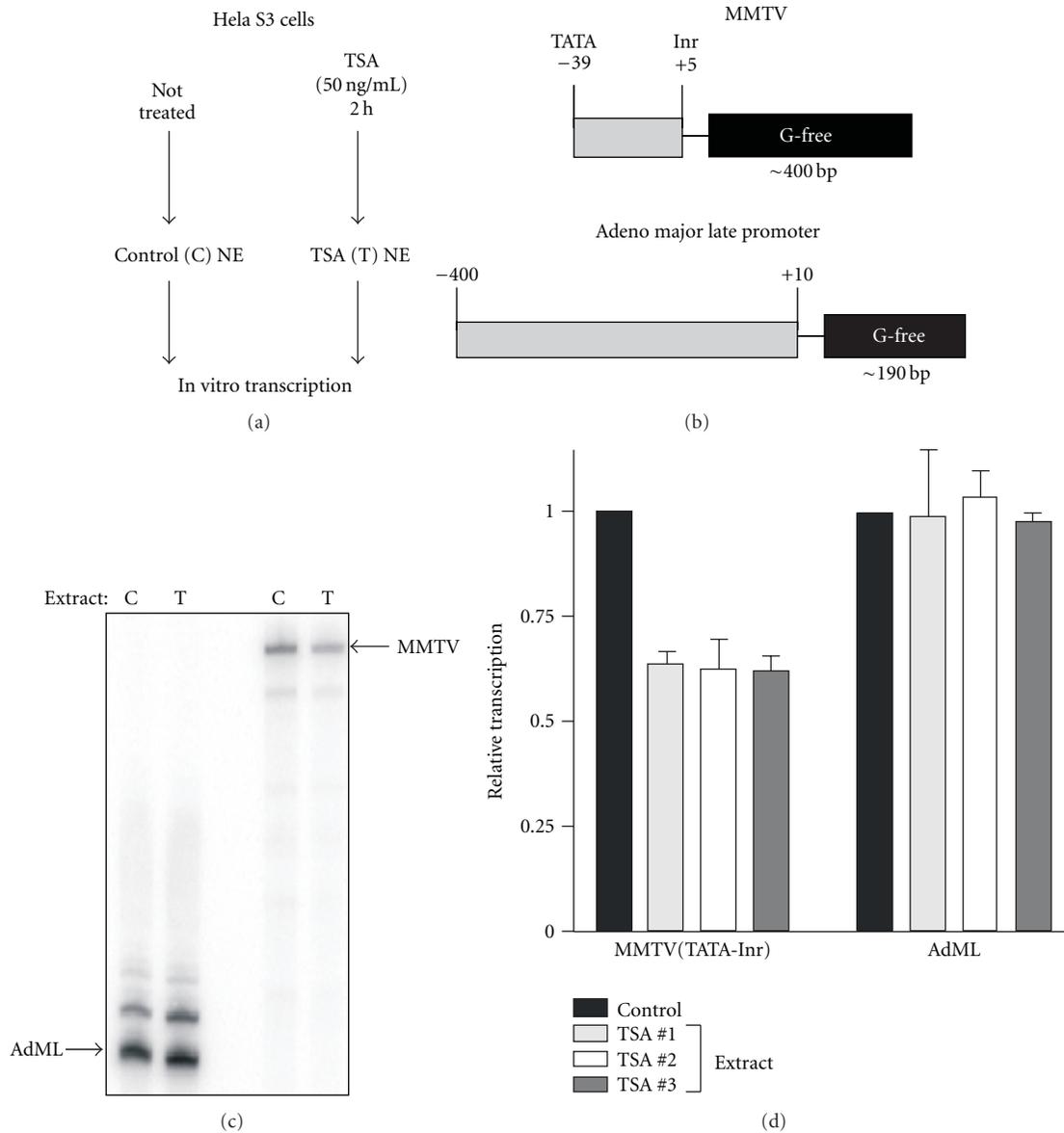


FIGURE 4: Repression of MMTV transcription by TSA is consistently recapitulated in an in vitro transcription assay. (a) Flow chart for a typical experiment. Nuclear extracts are generated from HeLa S3 cells which were either untreated or TSA treated. These extracts were used for in vitro transcription assays with the templates shown in (b). The MMTV template contains the region from the TATA box to the initiator element and a G-free cassette of 400 bp. The adenovirus major late (AdML) template contains a promoter fragment from -400 bp to +10 bp with a G-free cassette of 190 bp. Results of a typical transcription assay are shown in (c). C-extract from untreated cells (Control). T-extract from TSA-treated cells. (d) shows a graphic representation of assay results using three independent sets of extracts. Each extract was assayed with each template at least three times. Transcription levels in the extracts from TSA-treated cells are expressed relative to those in the corresponding extracts from untreated (control) cells.

in regulation of these distinct initiation processes at the MMTV core promoter, we first established the extent to which reinitiation occurs in our in vitro transcription system. The MMTV or AdML templates were incubated with nuclear extracts from untreated cells to allow de novo assembly of the PIC. Nucleotide triphosphates (NTPs) were added to initiate transcription. After 30 seconds, sarkosyl or water was added and transcription was allowed to proceed for 1 h. As shown in Figure 5(b), about 90% of the transcription product from

both templates is due to reinitiation since levels of transcript from each template in the presence of 0.05% sarkosyl were only 10% of those measured in its absence.

To determine whether HDAC inhibition impairs de novo transcription, the levels of MMTV transcript were measured after transcription in extracts from both control and TSA-treated cells in the presence or absence of sarkosyl. In Figure 5(c), transcript levels are expressed relative to those measured in control extracts in the absence of sarkosyl. As

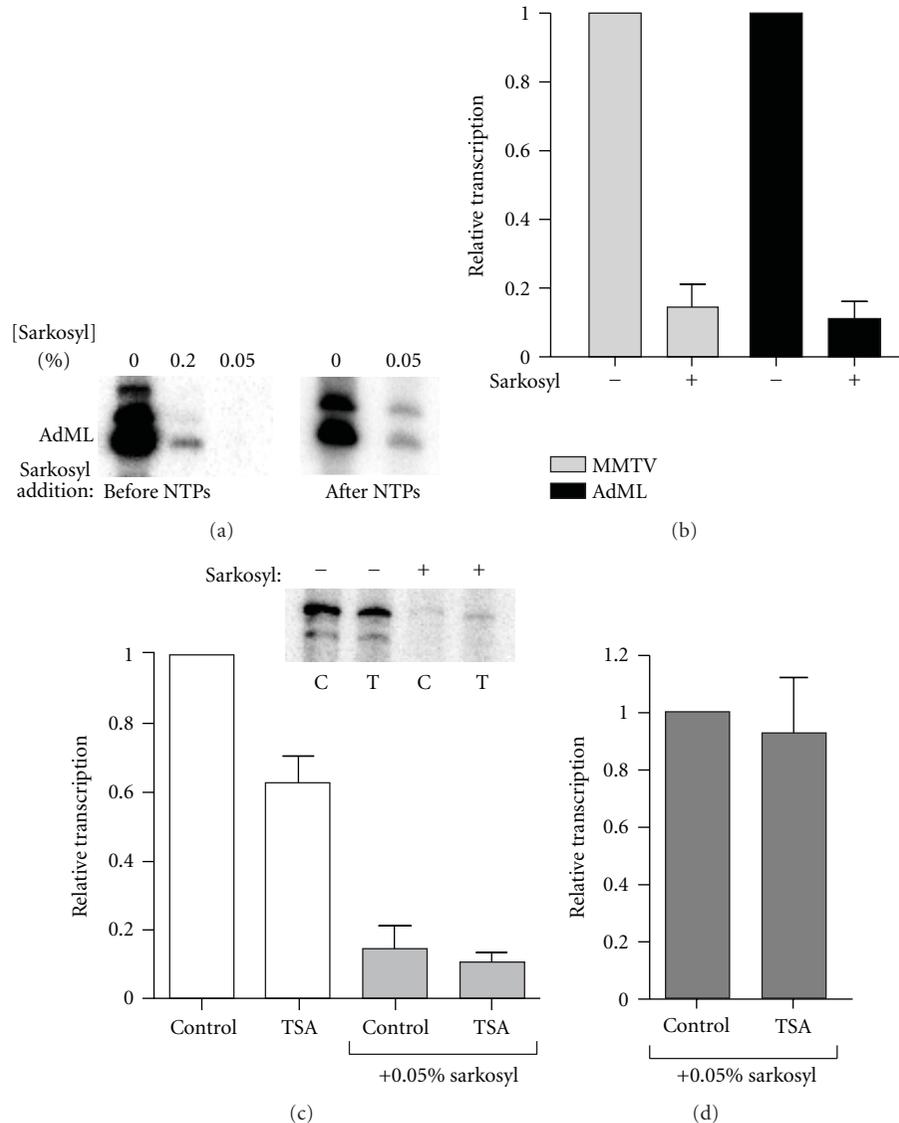


FIGURE 5: HDAC inhibition by TSA impairs reinitiation of transcription from the MMTV promoter in vitro. (a) In vitro transcription was carried out with the AdML template. Nuclear extracts were mixed with the template, and PIC assembly was allowed to proceed for 1 h. Sarkosyl was added either 2 minutes before or 10 seconds after NTP addition. Transcription was allowed to proceed for 1 h. (b) In vitro transcription was carried out in the presence or absence of sarkosyl (0.05%) added after NTP addition using either the MMTV or AdML templates as described in Section 2. Transcription levels in the presence of sarkosyl are expressed relative to levels of transcription measured in the absence of sarkosyl. (c, d) Transcription of the MMTV template was carried out using extracts from either control or TSA-treated cells in the presence or absence of sarkosyl (0.05%). In (c), transcription levels are expressed relative to those measured in control extracts in the absence of sarkosyl. In (d), transcription levels measured in extracts from TSA-treated cells are expressed relative to those measured in control extracts, both in the presence of sarkosyl. The results shown were obtained from three independent experiments, and error bars represent SEM.

expected, transcription of the MMTV template is reduced in extracts from TSA-treated cells in the absence of sarkosyl. However, in the presence of sarkosyl, transcript levels are similar in the two extracts. In Figure 5(d), transcript levels produced in the extracts from TSA-treated cells in the presence of sarkosyl are expressed relative to those measured in the control extracts under the same conditions to confirm that there is no significant difference. These results show that de novo transcription is unaffected by the lack of HDAC activity and strongly suggest that the reduction in MMTV

transcription in the extracts derived from TSA-treated cells is due to impaired reinitiation.

3.5. Initiation Complex Stability and HDAC Inhibition.

Two potential mechanisms by which HDAC activity might regulate transcriptional reinitiation include (1) the destabilization and dissociation of the reinitiation scaffold in the absence of HDAC activity and (2) inhibition of the activity of the scaffold and/or its ability to recruit RNA pol II. To distinguish between these possibilities, we performed

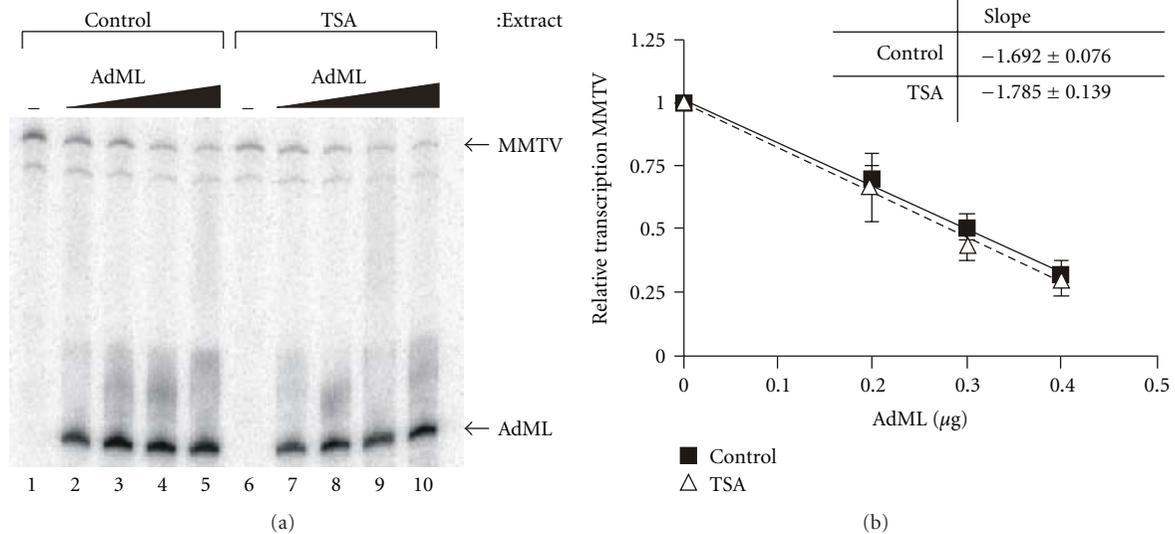


FIGURE 6: HDAC inhibition does not affect the stability of initiation complexes at the MMTV promoter as determined by template competition assay. Transcription of the MMTV template in extracts from control or TSA-treated cells was carried out in the presence of increasing amounts of AdML template. A representative experiment is shown in (a). (b) shows the relative level of MMTV transcription in extracts from either control or TSA-treated cells. Transcriptional levels in each extract are expressed relative to those measured in the absence of AdML template. The results shown are derived from at least 3 independent experiments. The data were subjected to linear regression analysis to generate lines and slopes using GraphPad Prism. Error bars represent SEM.

template competition assays in which transcription from a fixed amount of MMTV template was carried out in the presence of increasing amounts of the AdML template as a competitor. As the amount of AdML DNA increases, transcription from the MMTV promoter should decrease. This can be observed in Figure 6(a), lanes 1–5, and lanes 6–10 for transcription carried out in extracts from control and TSA-treated cells, respectively. The relationship between MMTV transcript levels and AdML template for each extract was then analyzed by linear regression to generate a line as shown graphically in Figure 6(b). If the lack of HDAC activity results in major destabilization of the reinitiation scaffold, the slope of the line depicting transcript levels in the extracts from TSA-treated cells would be significantly higher (steeper line) because the AdML template would compete more effectively for factors. However, the data shows that the slopes of the two lines are not significantly different even though the level of overall MMTV transcription in the extracts from TSA-treated cells is lower (shown in Figure 6(a)). This result suggests that the loss of HDAC activity does not affect the overall stability of the reinitiation scaffold but rather decreases its activity.

3.6. HDAC Activity Is Not Required during the Transcription Process. HDAC activity might regulate the efficiency of reinitiation either by being directly required during the process of transcriptional initiation or by regulating the activity of components of the reinitiation scaffold. In the case of the former, a critical deacetylation reaction may take place with every round of initiation, analogous to the way the CTD of RNA pol II must be dephosphorylated before it can reinitiate transcription. Alternatively, HDACs

regulate the activity of a scaffold component, perhaps through its acetylation status. Treatment of cells with HDAC inhibitors prior to generation of nuclear extracts would lead to the accumulation of this component in its functionally impaired state. This accumulation would thereby increase the chances of assembling functionally impaired scaffold complexes.

To distinguish between these possibilities, we asked whether TSA added to extracts from untreated control cells could inhibit MMTV transcription according to the scheme outlined in Figure 7(a). If HDAC action was required during each round of transcription, we expect that transcription would be inhibited by the addition of TSA to the control extract. To establish that the added TSA inhibits HDAC activity, an HDAC assay was performed to compare control extracts to which either TSA or DMSO had been added. As shown in Figure 7(b), HDAC activity is significantly inhibited by addition of TSA to a level even lower than that measured in extracts generated from cells treated with TSA. In vitro transcription was carried out on the MMTV templates using either extracts generated from cells treated with or without TSA or with the control extract to which TSA or the vehicle, DMSO, had been added. Consistent with previous experiments, MMTV transcription was reduced in extract from cells treated with TSA relative to control extracts. However, the addition of TSA directly to the control extract did not impair MMTV transcription. This result indicates that HDAC activity is not directly required during the initiation and elongation process and suggests that impaired function of the assembled basal transcription machinery is responsible for the reduced rate of transcriptional initiation.

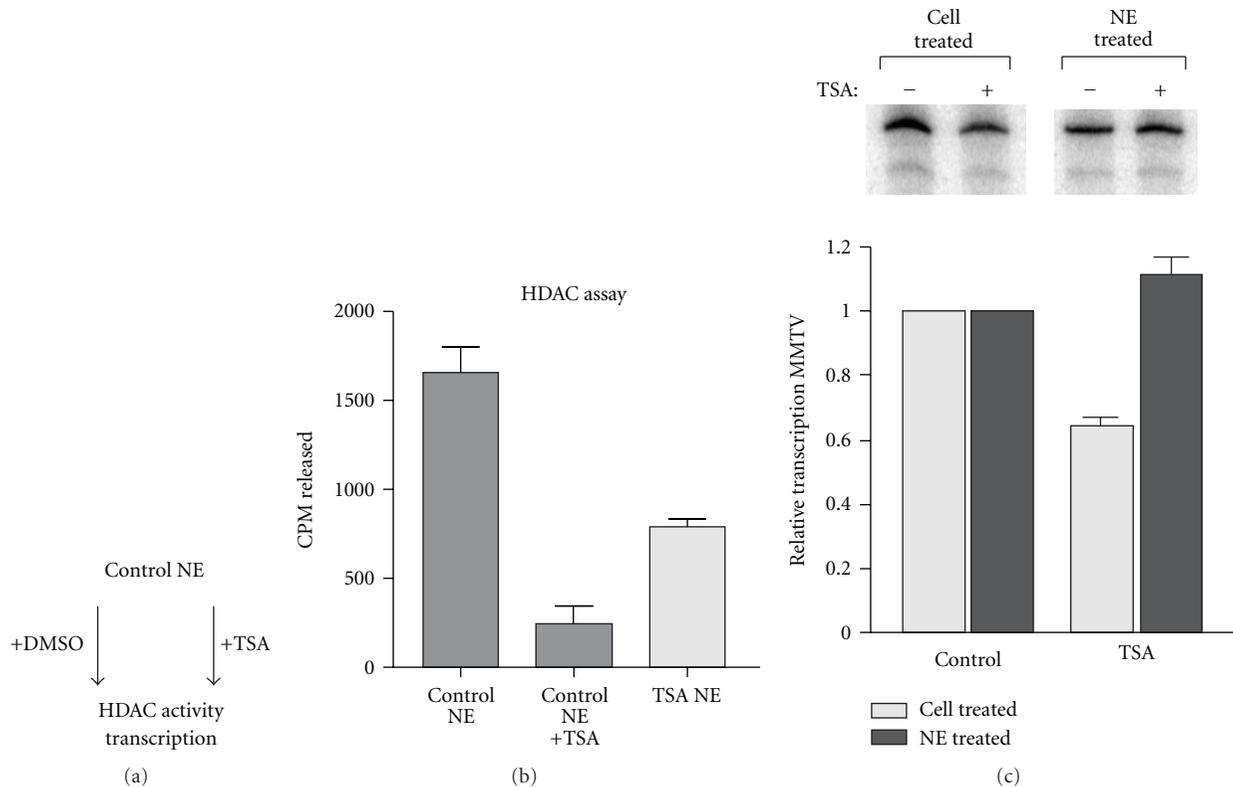


FIGURE 7: HDAC activity is not required during transcription of the MMTV promoter. (a) shows the experimental outline. Vehicle (DMSO) or TSA (50 ng/ml) was added to extracts from untreated (control) cells. These extracts were then subjected to HDAC assay or used for *in vitro* transcription. (b) shows the results of HDAC assays on control extracts to which vehicle or TSA had been added. The same assay was performed on extracts from cells treated with TSA as a comparison. The graph represents data from 3 independent experiments. (c) shows the results of *in vitro* transcription from the MMTV template comparing extracts from cells treated with or without TSA (Cell treated) with extracts from untreated (control) cells to which vehicle or TSA had been added (NE treated). The graph was generated from data derived from at least 3 independent experiments. Error bars represent SEM.

4. Discussion

Despite accumulating evidence that HDACs and protein deacetylation play positive roles in regulation of transcription, virtually nothing is known about the underlying mechanisms. In our previous study, we established that inhibition of HDAC activity caused repression of MMTV promoter activity that was transcriptional in nature and independent of changes in histone acetylation, chromatin structure, chromatin remodeling, and the binding of transcription factors upstream from the TATA box. In the current study, we have investigated the mechanisms behind this repression and found that it is mediated through the functional core promoter elements of the MMTV LTR and can also be observed with the core promoters of other genes. In addition, this repression is due to the inhibition of transcriptional initiation and correlates with decreased association of RNA pol II with the promoter. Development of an *in vitro* transcription system that recapitulates repression due to HDAC inhibition has allowed us to determine that reinitiation rather than *de novo* transcriptional initiation is sensitive to HDAC inhibition. The impairment of reinitiation is not due to wholesale destabilization of the scaffold

complex or a requirement for HDAC activity during each round of initiation, but rather, the results indicate that the ability of the assembled scaffold to recruit RNA pol II is inhibited. This study extends our knowledge of the transcriptional roles of HDACs and provides novel insights into the role of HDACs in facilitating transcription through the core promoter and basal transcription machinery.

Analysis of MMTV sequences necessary to confer repression on transcription through HDAC inhibition revealed its surprising dependence on the core promoter. The analogous region from several other genes displayed similar behavior. Interestingly, repression of both promoters of the *c-src* gene by HDAC inhibitors was found to be mediated through their core promoter regions [39, 40]. The core promoter is often viewed, not as an active participant in gene regulation, but only as a place to assemble the basal transcription machinery in response to the action of transcription factors bound to the promoter or distant enhancers. However, core promoter elements are not uniform between genes but usually occur in subsets (e.g., TATA/Inr, Inr/DPE, BRE/TATA/DPE) (reviewed in [30]). In addition, some transcriptional activators work more efficiently with a particular core promoter subtype, such as Caudal, a key regulator of Hox genes, which

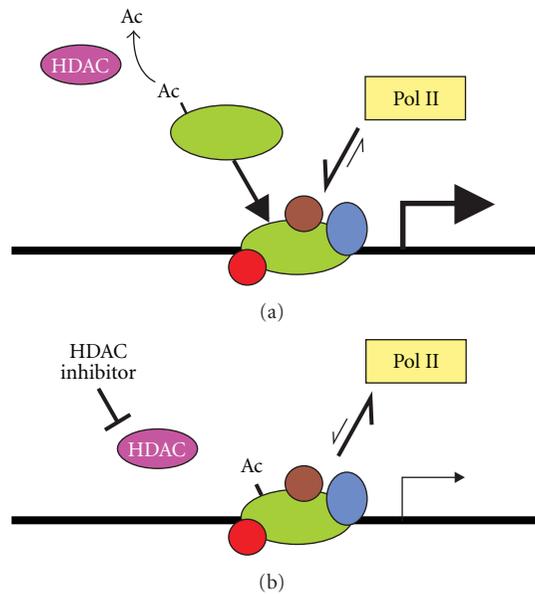


FIGURE 8: Proposed model for the role of HDACs in basal transcription from the MMTV promoter. (a) HDACs remove acetyl groups from proteins that are part of the reinitiation scaffold. This may occur prior to binding to the template (as shown) or at the template. This deacetylation facilitates binding of RNA pol II and the initiation of transcription. When HDAC activity is inhibited through HDAC inhibitors, shown in (b) persistent acetylation of basal transcription machinery components does not destabilize the complex or prevent its assembly. However, the recruitment of RNA pol II is impaired, leading to a decrease in the rate of transcriptional initiation.

prefers core promoters with a DPE to those with a TATA box [41, 42]. Such examples imply that the basal transcription complexes that form at these different core promoters differ somewhat in configuration, stability, or composition and therefore may respond distinctly to regulatory signals.

All the core promoters tested in our study contain both a TATA box and Inr element; some also contain an upstream BRE (CMV and CA2). Our results would seem to imply that all genes with TATA/Inr-containing core promoters are repressed by HDAC inhibitors, but we showed this not to be the case in our previous study [22]. We suggest that when transcription is strictly dependent on this type of core promoter, there is a requirement for HDAC activity. However, in the context of the other regulatory regions that control the expression of a particular gene, the combination of transcriptional regulators that bind to them can overcome this requirement. For promoters such as MMTV, the transcriptional regulators that bind upstream of the TATA box are unable to overcome the repression of basal transcription caused by the loss of HDAC activity. The gene specificity of the core promoter requirement for HDAC activity may be mediated by dependence of key transcriptional regulators on different components of the basal transcription complexes to facilitate transcriptional initiation. Some subunits of the Mediator and TFIID complexes have been shown to function in a gene-specific fashion and/or to be differentially utilized

by promoter-bound transcription factors (reviewed in [43, 44]). For example, the ligand-dependent nuclear receptors have been shown to require the presence of the Med1 subunit of the mediator complex to activate some target promoters, and the estrogen receptor (ER) has been shown to contact TAF10 of TFIID to activate transcription [45]. Interestingly, GR has been reported to require different mediator subunits in distinct promoter contexts [46, 47]. Posttranslational modification (such as acetylation) of complex subunits required for interaction with particular transcription factors may disrupt those interactions and lead to a reduction in activated or basal transcription. Alternatively, a transcription factor bound to a gene may bypass the negative effects of a functionally impaired subunit of the basal transcription machinery if it requires other subunits to facilitate transcription. In the case of MMTV, we propose that the upstream factors that drive transcription require the function of the impaired subunit(s) that causes repression of core promoter activity and therefore cannot overcome the loss of HDAC activity.

Our experiments with sarkosyl show that *de novo* transcriptional initiation from the MMTV promoter is unaffected by HDAC inhibition, indicating that the repression observed is due to impaired reinitiation. *De novo* initiation involves the assembly of the PIC at a completely inactivate promoter. Once the original round of transcription has begun and the polymerase leaves the core promoter, a scaffold is left behind which contains general transcription factors TFIID, -IIA, -IIH, -IIE, and the mediator complex [48]. Some transcription factors stabilize this scaffold and facilitate transcription by increasing the rate of reinitiation [48–51]. In a study of the effects of TSA on HIV transcription *in vitro*, it was observed that TSA increased the amount of *de novo* transcription [51]. These experiments were performed with chromatin-assembled HIV templates, and the stimulatory effect of TSA on *de novo* transcription may have been due to increased histone acetylation, which has been shown to facilitate interactions between transcription factors and chromatin (reviewed in [52]). The same study also showed that TSA had little effect on reinitiation of HIV transcription [51]. However, it was also determined that the transcription factors which bind the HIV promoter were highly efficient in stimulating reinitiation *in vitro*. As we discussed above, it is certainly possible that this combination of factors can overcome any negative effects of acetylation on reinitiation in the HIV promoter context. Our studies of the MMTV promoter strongly suggest that this is not true of the combination of transcription factors that control its transcriptional activity. However, the specific effects of HDAC inhibition on reinitiation versus *de novo* initiation provides a potential explanation for the ability of GR to partially activate the MMTV promoter in the presence of TSA or HDAC1 depletion [17, 22]. There is a level of basal MMTV transcription in our cell lines, which is likely to be due to either stochastic *de novo* assembly of the PIC and/or a low rate of reinitiation from pre-existing scaffold complexes. GR may act to both promote *de novo* assembly of the PIC at MMTV templates within a cell population and increase reinitiation at preassembled scaffold complexes. Our results

suggest that GR-induced de novo transcription within a cell population would not be affected by loss of HDAC activity but that reinitiation would be impaired, thus decreasing the magnitude of GR-activated MMTV transcription but not completely inhibiting it.

Why would HDAC activity and deacetylation affect only reinitiation and not de novo transcription? A recent study indicates that the structure of the reinitiation scaffold is distinct from that of the initial PIC. Knuesel and colleagues showed that the Cdk8 submodule of the mediator can bind to the reinitiation scaffold but not the initial PIC [53]. Once bound, it can repress the rate of reinitiation because RNA pol II cannot rebind. They hypothesize that there is a structural shift in mediator structure that occurs once pol II has cleared the initial PIC which allows this module to bind and act as a rheostat. It is also possible that conformational changes occur in other components of the basal transcription machinery after the initial round of transcription. A difference between the initial PIC and the reinitiation scaffold may also explain the differential ability of transcription factors to facilitate reinitiation [48]. Our study has elucidated another key difference between reinitiation and de novo transcription and that is a requirement for HDAC activity.

How does HDAC activity affect reinitiation? The template competition experiments indicate that HDAC activity does not stabilize the reinitiation scaffold since the ability of the AdML template to compete with MMTV for complex assembly was not increased in extracts from TSA-treated cells. This finding implies that it is the activity rather than the overall stability of the scaffold that is impaired when HDAC activity is compromised. The ChIP experiments showed that TSA treatment resulted in decreased association of RNA pol II with the MMTV promoter. Thus, it is possible that the ability of the reinitiation scaffold to recruit pol II is dependent on HDAC activity as shown in the model depicted in Figure 8. However, inhibition of HDACs through addition of TSA to the control nuclear extracts did not lead to inhibition of MMTV transcription, meaning that HDAC activity is not required during the process of reinitiation, perhaps removing acetyl groups that were acquired during initiation and must be removed prior to binding of another pol II complex, analogous to the phosphorylation cycles of the pol II CTD [34].

We hypothesize that treatment of cells with TSA prior to generation of the nuclear extracts led to changes in the acetylation status of one or several proteins that regulate transcriptional initiation. This regulation could be indirect, involving HDAC target proteins that influence the activity or post-translational modification of the basal transcription machinery but are not directly involved in transcriptional initiation at the core promoter. However, we argue that the effect of acetylation on transcriptional initiation is more direct. First, nuclear run-on experiments in our previous study clearly showed that basal transcription from the MMTV promoter decreases immediately, within 5 minutes of TSA treatment [22]. A delay might be expected if an indirect mechanism was in play. Second, it has been shown that HDAC1 is constitutively present at the MMTV promoter

and that its depletion by RNA interference impairs MMTV transcription [17]. A change in its activity by exposure to HDAC inhibitors could immediately affect the dynamics of acetylation at the promoter to cause a rapid decrease in transcription through increased acetylation of proteins at the promoter. These proteins could be subunits of the complexes that make up the reinitiation scaffold or proteins known to directly regulate basal transcription such as NC2, BTAF, or the Cdk8 module of the mediator complex (reviewed in [43, 54]). Loss of HDAC activity would increase the acetylated fraction of these proteins, thereby increasing the chance that acetylated forms were incorporated into PICs and scaffolds formed at our templates in vitro. HAT activity in the nuclear extracts may not have been robust enough or targeted enough to significantly increase acetylation of these proteins when HDACs were inhibited in vitro (Figure 7). In spite of the fact that TFIID contains an acetyltransferase (TAF1) and robust HATs such as CBP, p300, and PCAF are recruited to many promoters, very little is known about acetylation of the basal transcription machinery, including whether there are acetylated subunits, and if so, how acetylation affects their function.

In summary, our study has uncovered a novel mechanism by which HDACs function in facilitating transcriptional initiation. Future studies will be directed at identifying the critical target of HDAC activity that regulates basal transcription and further dissection of the mechanism by which HDAC activity impacts reinitiation.

Acknowledgments

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Research Article

Identification of Four Potential Epigenetic Modulators from the NCI Structural Diversity Library Using a Cell-Based Assay

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Epigenetic pathways help control the expression of genes. In cancer and other diseases, aberrant silencing or overexpression of genes, such as those that control cell growth, can greatly contribute to pathogenesis. Access to these genes by the transcriptional machinery is largely mediated by chemical modifications of DNA or histones, which are controlled by epigenetic enzymes, making these enzymes attractive targets for drug discovery. Here we describe the characterization of a locus derepression assay, a fluorescence-based mammalian cellular system which was used to screen the NCI structural diversity library for novel epigenetic modulators using an automated imaging platform. Four structurally unique compounds were uncovered that, when further investigated, showed distinct activities. These compounds block the viability of lung cancer and melanoma cells, prevent cell cycle progression, and/or inhibit histone deacetylase activity, altering levels of cellular histone acetylation.

1. Introduction

Pathologies such as human cancer result largely from the inappropriate silencing or activation of genes. It is well established that gene expression can be partly controlled by modulating the access of the transcriptional machinery to target genes through chemical modifications of DNA sequences or histones, the proteins that package DNA. These modifications are mediated by cellular enzymes, including DNA methyltransferases, histone acetyl transferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), histone demethylases, and histone kinases. Epigenetic enzymes function during development, helping orchestrate complex transcriptional programs that control differentiation pathways. During adult life, these enzymes continue to influence transcription by maintaining tissue-specific epigenetic and transcriptional patterns as well as by acting as coregulators for transcription factors. In many cancers, the regulation of transcriptional processes is altered partly because of the abnormal expression and/or function of epigenetic enzymes resulting in the silencing

of tumor suppressor genes or in equivalent events that lead to unchecked cellular growth [1]. Indeed, it has been estimated that epigenetic changes are at least ten to forty times more frequent in cancers than genetic mutations [1–3]. Furthermore, specific mutations in HATs and HMTs have been found in relation to the cancer phenotype, suggesting them as potential targets for therapy [4]. Thus, effective treatment of cancer will, at least in part, necessitate the chemical targeting of the cancer epigenome.

Over the last two decades, drugs have been identified that modulate the pathways mediated by a subset of epigenetic enzymes. Because cancer cells often have abnormally silenced tumor suppressor genes or overexpressed oncogenes, mediated by epigenetic pathways, these drugs have been studied in preclinical contexts. Of the known compounds, however, only a few have shown success in clinical settings, with toxicities observed for most other compounds due to their global, unspecific effects on cell function [5–9]. This has led to new drug discovery and drug development efforts at industrial and academic laboratories over the last few years. These programs have used cell-based, *in vitro*, *in silico*,

or yeast systems to identify novel drugs or have developed second-generation compounds structurally related to already known inhibitors [10–18].

Altogether, more recent studies have yielded several new compounds that target epigenetic enzymes, primarily histone deacetylase family members and enzymes that modulate methylation [15, 19–22]. Some of these compounds offer limited benefit over existing drugs, since they are structurally closely related to known inhibitors of epigenetic enzymes, are unspecific, or lack substantial *in vivo* activity, due at least in part to limitations in drug screen design. To date, there are only a few epigenetic drugs approved by the FDA, including: 5-azacytidine and its deoxy derivative decitabine, both DNA methyltransferase inhibitors used for the treatment of myelodysplastic syndromes, vorinostat, and recently romidepsin, HDAC inhibitors used for the treatment of cutaneous T cell lymphoma [23–26]. There is, therefore, a persistent need to increase the number and diversity of available anticancer epigenetic modulators and to develop innovative, improved approaches for drug discovery.

Because screens that use *in vitro* or *in silico* approaches may lead to hits that prove to be toxic, insoluble, or inefficient when taken to the *in vivo* setting, using systems in which drugs are directly tested in cells, as was done for some of the original HDAC inhibitors [10, 11, 18], can save time and effort on followup studies of drugs that are only effective *in vitro* or would require substantial chemical optimization. Here, we report the characterization and use of a cell-based assay in which a locus containing an easily quantifiable marker, green fluorescent protein (GFP), is epigenetically silenced, and derepressed chemically by known epigenetic modulators targeting both histone acetylation and DNA methylation. We have now successfully used this system, the Locus Derepression assay (LDR) [27, 28], to screen the NCI's structural diversity library to identify novel compounds with epigenetic activity. Four confirmed hits from the screen were further investigated for their anticancer properties and their ability to inhibit histone deacetylases. We found that two of our hits potently blocked the viability of both lung cancer and melanoma cells and that one of them caused cancer cells to accumulate in the G2/M phase, preventing cell cycle progression. A third hit inhibited deacetylase activity *in vitro* and in cells, but on its own had little toxicity, while a fourth compound selectively inhibited the viability of melanoma cells compared to lung cancer cells.

2. Materials and Methods

2.1. Chemicals and Reagents. Dulbecco's modification of eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), and G418 were purchased from Mediatech (Manassas, VA). The penicillin/streptomycin solution, trypsin-EDTA, and 37% formaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). Hoechst 33342 was obtained from Invitrogen (Carlsbad, California). Trichostatin A and apicidin were obtained from Alexis Biochemicals, decapeptide was a generous gift from Dr. David Schrupp and 5-aza-2'-deoxycytidine and sodium butyrate were purchased from Sigma/Aldrich. Hit compounds for followup were provided

by the Drug Synthesis and Chemistry Branch, DTP, NCI. All drugs were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C .

2.2. Cell Culture and Materials. LDR cells were grown in DMEM media with 10% heat inactivated FBS and supplements, as previously described [29]. The C127 cells were grown in DMEM supplemented with 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 10% charcoal/dextran-treated FBS. Cells were cultured in a humidified incubator at a 37°C , 5% CO_2 , and 95% air environment.

2.3. Drug Screen. LDR cells were plated into 96-well Nunc glass-bottom black plates at 8,000 cells/well and cultured in complete media at 37°C , 5% CO_2 , and 95% air overnight. DMSO (0.5%) was used for the negative control and 25 mM sodium butyrate was used as the positive control. Twenty-four hours after seeding the cells into assay plates, cells were treated with the structural diversity chemical library (4 μM final). After a 24 hour treatment with the chemical library, the assay plates were subjected to fixation with 4% formaldehyde for 45 minutes. After fixation, the assay plates were washed 5 times with 100 μL of DPBS. The plates were stained with 0.3 $\mu\text{g}/\text{mL}$ Hoechst 33342 in DPBS overnight at 4°C . The next day, assay plates were washed twice with 100 μL of DPBS, and the plates were sealed with aluminum sealing tape and were barcoded with a Velocity-11 VCode Bar Code Label Print and Apply Station (Menlo Park, CA). Images were acquired using the Discovery-1 imaging system, at 20X, 4 sites per well, 30 ms exposure for Hoechst 33342, and 600 ms exposure for GFP, and they were processed with Metamorph software. Hits were identified by manual examination of images for GFP expression. Hit compounds were cherry picked and subjected to quadruplicate testing in the LDR cell line and in the C127 parental cell line to confirm activity and eliminate false positive results due to fluorescent compounds.

2.4. Fluorescence Microscopy. For followup experiments, LDR cells were plated on four-well chambered glass slides (Lab-TekII, Nunc) at a density of 15,000 cells per well, treated with drug and incubated for 24 hours in 5% CO_2 at 37°C , and imaged either on a Nikon Eclipse TE2000-U fluorescence microscope equipped with a CCD Roper camera, an Olympus 1 \times 70 system equipped with a Photometrics camera or on a CARV Metamorph system with an Orca II CCD camera. Metamorph software was used to process the images.

2.5. GFP Quantification. Fluorescent Activated Cell Sorting (FACS) was used to quantify GFP levels in LDR cells after various drug treatments in dose response studies. Briefly, cells were collected and pelleted, washed in PBS, and then resuspended in PBS at 100,000 cells/mL and subjected to analysis in BD FACSCalibur sorter. Data was analyzed using FlowJo software. Uninduced and vehicle treated cells were used as a negative control for gating.

2.6. Cell Viability Assays. Human cancer cell lines were plated in RPMI media with 5% heat-inactivated FBS, at 2000–4000 cells per well of 96-well flat-bottom plates. The next day, cells were treated with compounds with maximum concentrations ranging between 15 μ M and 5 μ M and allowed to incubate for 4 days in 5% CO₂ at 37°C. Cells were then treated with MTS reagent and absorbance measured according to the company's protocol (CellTiter 96 AQueous Nonradioactive Cell Proliferation Assay, Promega).

2.7. Cell Cycle Analysis. H358 cells were treated with 1 μ M 5-aza, 200 nM TSA, 5 μ M NSC-159631, or DMSO and incubated for 24 hours in 5% CO₂ at 37°C. Cells were trypsinized and stored in 75% ethanol at –20°C and later washed in PBS and suspended in propidium iodide staining buffer (1x PBS, 0.1% Triton X-100, 0.5 mM EDTA, 50 μ g/mL DNase-free RNase, and 0.05 mg/mL propidium iodide). Samples were then analyzed in the UT Southwestern FACs core using a BD FACSCalibur sorter. Data was analyzed with FlowJo software.

2.8. HDAC Assays. HDAC activity was analyzed using the HDAC Assay Kit from Millipore/Upstate (17-356). Briefly, nuclear extract from Baf3 cells or purified, recombinant HDAC1 (Millipore/Upstate) was allowed to incubate with the fluorometric HDAC substrate according to the manufacturer's protocol. In a secondary activator reaction, the fluorophore is only cleaved from the deacetylated substrate, allowing for quantification. Fluorescence was quantified on a FLUOstar-Optima or a FLUOstar Omega plate reader (BMG Biosciences).

2.9. Western Blot Analysis. H358 cells were treated with 10 μ M NSC-22206, 200 nM TSA, 0.5 μ M NSC-159631, or DMSO for 24 hours, and protein was extracted using a lysis buffer (50 mM NaCl, 1 mM EDTA, 2.5 mM Tris pH 7.4, 0.1% SDS, and 1% NP-40). Protein was quantified and equal amounts of samples were run on 4%–12% SDS acrylamide gradient gels and transferred to nitrocellulose membranes. Membranes were incubated overnight with 0.05 μ g/mL polyclonal rabbit anti-acetyl-Histone 3 antibody (Millipore/Upstate 06-599). Blots were washed and re-incubated with anti-actin primary antibody (Santa Cruz no. 1616). Bands were imaged using enhanced chemiluminescence reagents from Thermo Scientific. For Supplementary Figure 2B available at doi: 10.1155/2011/868095, the additional antibodies used were as follows: HDAC1 (Affinity Bioreagents PA1-860), acetylated tubulin (Sigma T6793) and H3K9me3 (Millipore 07-523).

3. Results and Discussion

3.1. Development of Cell-Based GFP Assay. To screen for epigenetic modulators, we developed a cell-based assay, consisting of C127 mouse mammary adenocarcinoma cells stably expressing a GFP construct linked to a portion of the estrogen receptor ligand binding domain, driven by the CMV promoter, which is susceptible to epigenetic repression. On a separate plasmid, the gene for neomycin resistance was also

introduced. We selected cells that were neomycin resistant but whose GFP expression was constitutively silenced. After clonal expansion, GFP expression remained repressed. Upon treatment of cells with epigenetic modulators, we expected GFP production (Figure 1). The use of GFP allows for easy and automatic detection, applicable to both low and high throughput screening applications. The detailed design of this cell-based assay, named the locus derepression or LDR assay, has been described elsewhere [27, 28].

3.2. Characterization of the LDR Assay. To determine if the GFP construct in LDR cells was under epigenetic regulation and if so, what classes of epigenetic modulators would derepress the locus, LDR cells were treated with different structural classes of HDAC inhibitors and with DNA methyltransferase inhibitors. The cells were then analyzed for GFP expression by visualization with a fluorescent microscope (Figure 2(a)), evaluated in dose-response experiments (Figure 2(b)) and/or quantified by fluorescence-activated cell sorting (FACS) (Figure 2(c)). Class I/II HDAC inhibitor trichostatin A (TSA), a hydroxamic acid, was added to the cells at a concentration of 165 nM, and GFP expression was measured. Treating cells with increasing concentrations of TSA ranging from 16 nM to 827 nM demonstrated that the induction of GFP in LDR cells was dose dependent (Figure 2(b)). The short chain fatty acid butyrate, also a Class I/II HDAC inhibitor, was able to induce expression of GFP as measured by both microscopy (Figure 2(a), middle left panel) and FACS (Figure 2(c)). Butyrate induction of GFP expression was also seen to be dose dependent when tested with concentrations ranging from 1 mM to 100 mM (Figure 2(b)). Similar results were observed with apicidin, a cyclic tetrapeptide HDAC inhibitor. Cells treated with 5 μ M apicidin had a significantly increased green fluorescent population, as shown by FACS (Figure 2(c)). Apicidin also derepressed the GFP construct in a dose-dependent manner, at concentrations between 0.1 μ M and 10 μ M (Figure 2(b)). We also evaluated a Class I-specific HDAC inhibitor, the cyclic depsipeptide FR901228 also known as simply depsipeptide or romidepsin, which potently induced GFP production at 25 ng/mL (Figure 2(a), bottom left panel). Taken together, these results demonstrate that the GFP construct in LDR cells is under epigenetic regulation which can be overcome by any structural type of HDAC inhibitor. As expected from their functionally distinct roles, inhibition of class 3 HDACs by nicotinamide had no effect on GFP production although Sirt 1 enzymatic activity was markedly diminished in vitro (data not shown). Interestingly, BIX-01294, an inhibitor of G9a histone methyltransferase [15], did not induce GFP expression at up to 10 μ M doses over a two day exposure, suggesting that histone methylation by G9a is not required for CMV silencing in LDR cells.

To test whether the silenced locus was also under the control of DNA methylation (which was possible due to the presence of a large CpG island on the CMV promoter), we measured the effects of a DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-aza) and found that it too was able to induce GFP expression (Figure 2(a), bottom right panel). Similar results were obtained with 5-aza-cytidine.

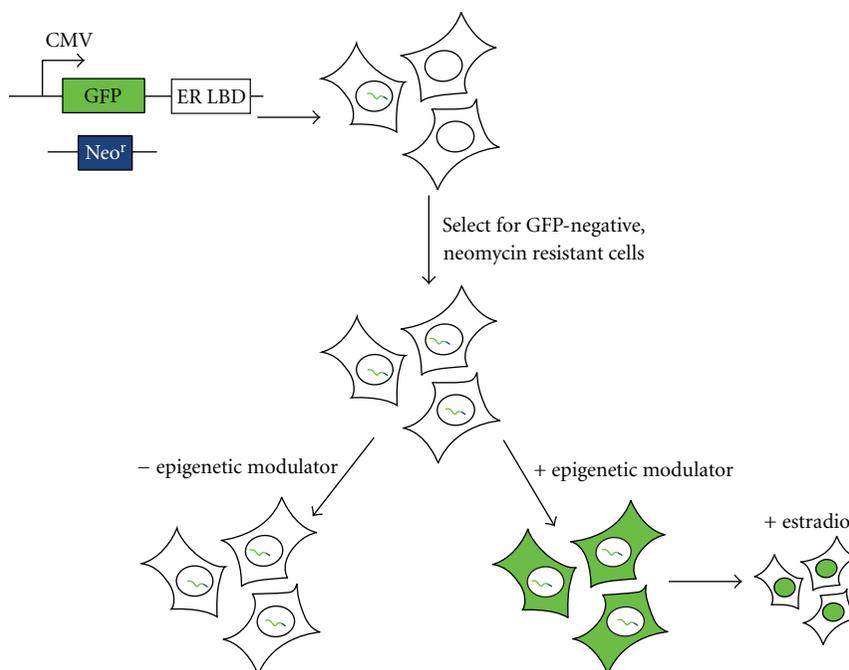


FIGURE 1: Schematic representation of the process of creating transgenic mouse mammary adenocarcinoma cells (locus derepression assay cells or LDR) [29]. A construct containing the GFP gene linked to the estrogen receptor ligand-binding domain, driven by the CMV promoter and a neomycin resistance marker, was stably transfected into cells. GFP negative cells, stably expressing the neomycin resistant gene, were selected. With the addition of an epigenetic modulator, such as an HDAC inhibitor or a DNA methyltransferase inhibitor, the cells express GFP and can be visualized by fluorescence microscopy. When cells are then treated with estradiol, the GFP fusion protein translocates to the nucleus.

However, other molecules, such as general transcriptional activators including hormones, steroids, and amino acids, were incapable of inducing GFP production, as were proteasome inhibitors or general stress conditions such as hypoxia and starvation (data not shown). These experiments show that the GFP construct is, indeed, silenced epigenetically and can be derepressed by inhibiting HDAC as well as DNA methyltransferase pathways (and potentially other epigenetic cascades), emphasizing the assay's utility in finding novel epigenetic modulators.

To confirm that GFP expression induced by drug treatment was dependent upon *de novo* transcription of the locus and did not, for example, involve increased stability or translation of an already transcribed message, we measured the effects of actinomycin-D on GFP production. We cotreated LDR cells with 200 nM TSA or 25 nM depsipeptide, in the presence or absence of 0.5 $\mu\text{g}/\text{mL}$ actinomycin-D, a known inhibitor of transcription. Actinomycin-D prevented the production of GFP in response to both TSA (Figure 3(a) and 3(b)) and depsipeptide (Figures 3(c) and 3(d)). This confirms that the expression of the silenced locus requires new transcription, engaging the pathways the assay was designed to exploit.

3.3. Drug Screens Using the LDR Assay and Characterization of Hit Molecules. To adapt LDR cells for use in screening applications, we first isolated a subset of the most responsive transgenic cells, which showed a 70%–90%

response to 25 mM butyrate (Figure 4(a)) and expanded them clonally. These cells grew well in 96-well plate format and produced robust GFP signal (Figure 4(b)). We used these LDR cells for drug screens to identify potential novel epigenetic modulators that would turn on the expression of GFP in cells. Cells were plated in 96-well plates, grown overnight, and treated for 24 hours with 4 μM concentrations of compounds from the NCI structural diversity library, which was chosen in order to maximally query chemical space for new active structures. After fixation, cells were visualized using a Discovery-1 automated fluorescent microscope which captured two-by-two frames in each well (Figure 4(b)). Hits were manually scored for GFP expression, and of the 2,080 compounds screened, 71 of them were primary hits. However, after eliminating the autofluorescent compounds by counter screening on parental C127 cells, and retesting the remaining hits through cherry picking, there were 4 confirmed hits which were followed up (Table 1). These confirmed hits were validated at the protein level by measurements of GFP protein translocation in response to estradiol, making use of the estrogen receptor ligand-binding domain which is expressed in LDR cells downstream of GFP on the same construct (see Supplementary Figure 1) [28].

The hits from this library are not only structurally distinct from each other, but also from other known HDAC and DNA methyltransferase inhibitors, providing structural diversity to this chemical class. To analyze if these compounds were active at a single dose or showed

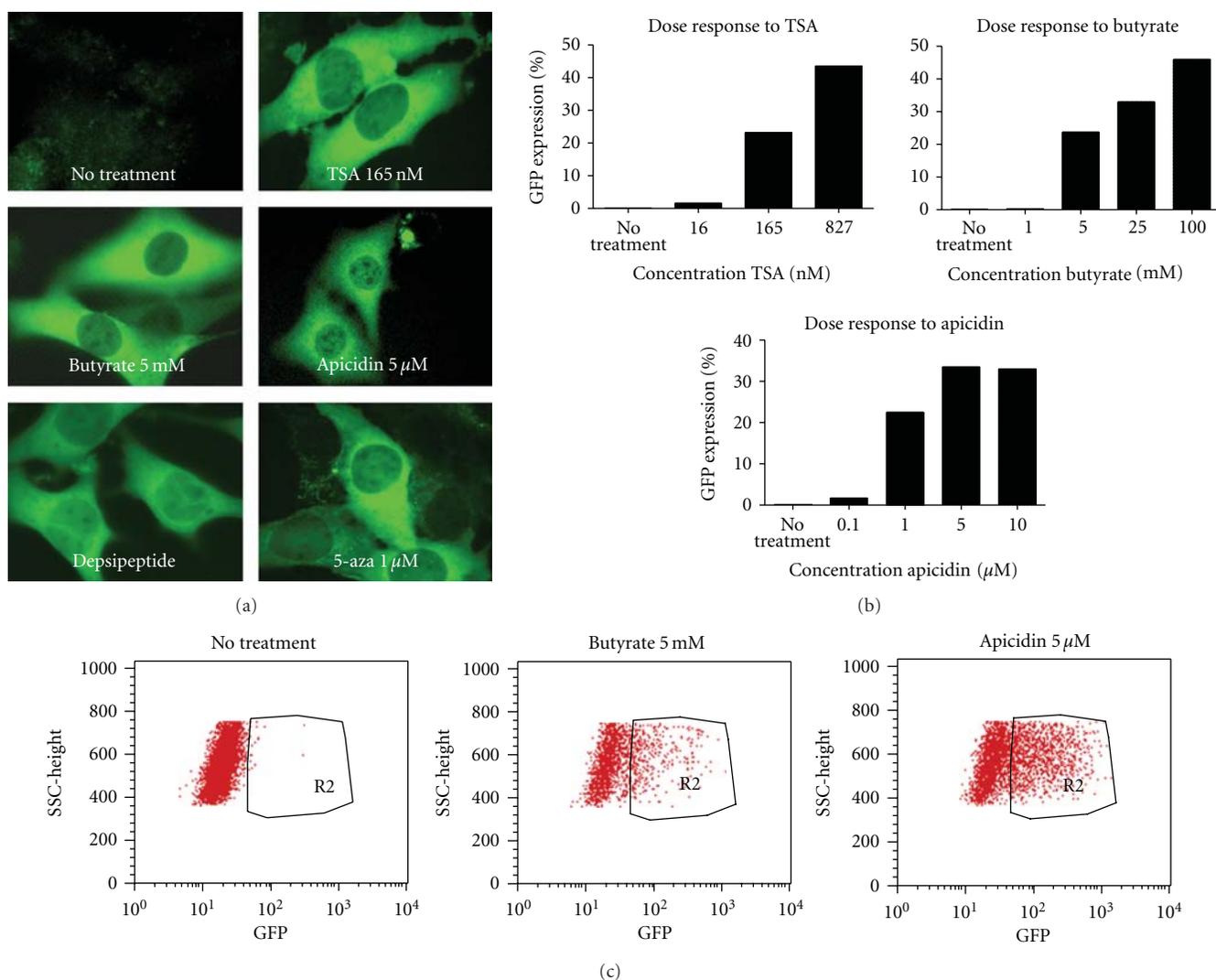


FIGURE 2: Characterization of cell-based LDR assay. (a) Visualization of GFP expression in cells treated with various known epigenetic modulators. TSA, butyrate, apicidin, and depsipeptide are HDAC inhibitors and 5-aza-deoxycytidine is a DNA methyltransferase inhibitor. (b) Cells exhibit dose-dependent GFP expression in response to HDAC inhibitors. (c) FACS analysis reveals an increase in number of cells expressing GFP when treated with butyrate or apicidin, compared to untreated cells.

dose responsiveness, two of our hits were analyzed in a concentration series. Secalonic acid D (NSC-159631) [30], was added in concentrations ranging between 10 nM and 10 μ M, and NSC-22225 was tested between 500 nM and 10 μ M. GFP induction in response to secalonic acid D was strongly dose dependent showing a half-maximal activation between 1–5 μ M (Figure 5(a)), while NSC-22225 showed a weaker but nonetheless clear dose dependence (Figure 5(b)), note toxicity to mouse LDR cells at 10 μ M). A search for compounds structurally related to our hits, yielded NSC-22206 and NSC-22214, which vary from NSC-22225 only in the nature of the metal salt. As expected, these related molecules also induced GFP expression in LDR cells but had a slightly lower potency than the primary hit (Figure 5(c) and data not shown).

Epigenetic modulators have been postulated to have anticancer properties because they can mediate the

reexpression of silenced tumor suppressor genes and in a manner, that is not yet fully understood, can block cell cycle progression [1, 18, 22, 31, 32]. To evaluate the ability of LDR hits to inhibit the viability of human cancer cells, we performed standard MTS assays on non-small cell lung cancer and melanoma cells [33, 34]. Table 2 shows the average IC_{50} values obtained in 1–5 experiments each done with 8 replicates. While NSC-22225 and its related compound were incapable of blocking human cancer cell growth, other hits inhibited cancer cell viability with IC_{50} 's ranging from 90 nM to 9 μ M. Interestingly, while NSC-150117 and secalonic acid D were effective against both lung cancer and melanoma cells of uveal origin, NSC-693322 preferentially targeted melanoma cells, suggesting that this compound may interfere with a signaling cascade essential for melanoma but not lung cancer cell survival. Secalonic acid D, known to have activity as a kinase inhibitor, was the hit with the broadest activity

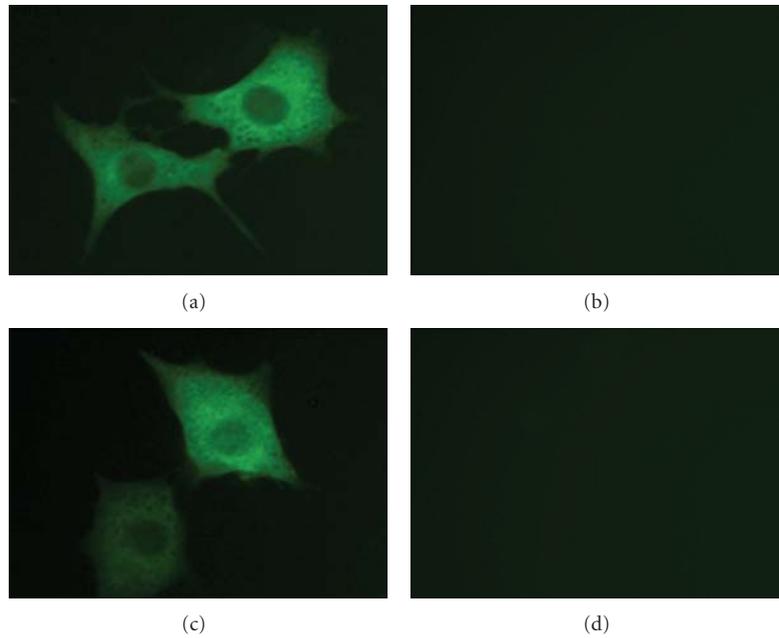


FIGURE 3: GFP expression requires *de novo* transcription. (a) Cells treated with 200 nM TSA express GFP. (b) When transcription inhibitor actinomycin-D ($0.5 \mu\text{g/mL}$) is added to LDR cells together with TSA, no GFP expression is observed. The same pattern is seen with 25 nM depsipeptide, with and without actinomycin-D in (c) and (d), respectively.

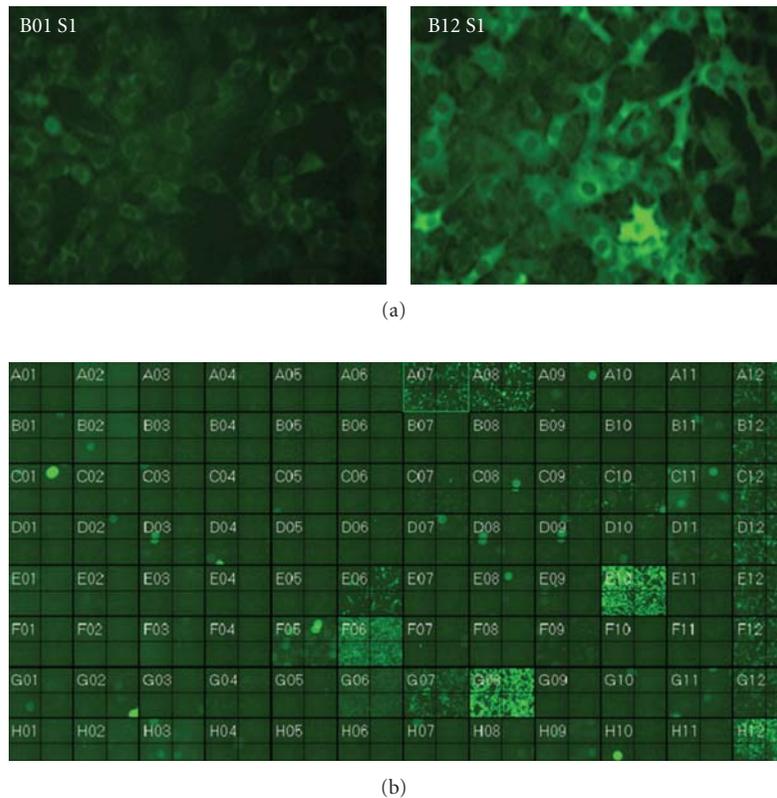
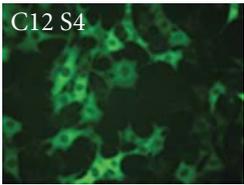
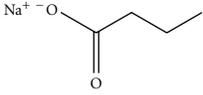
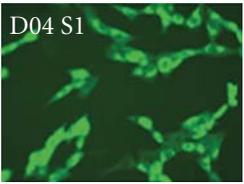
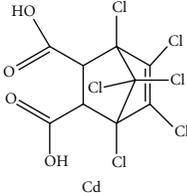
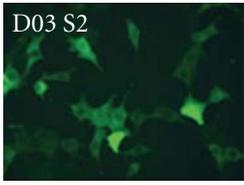
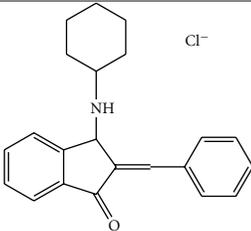
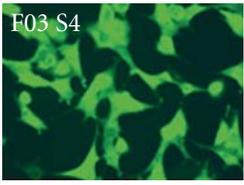
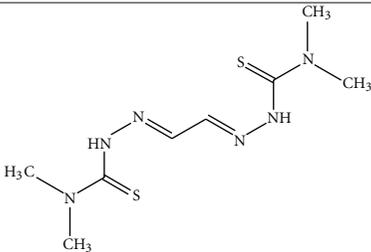
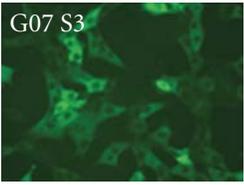
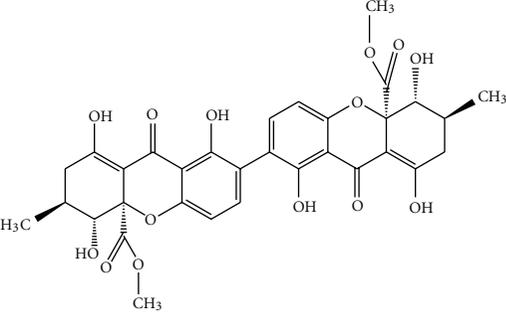


FIGURE 4: Transgenic LDR cells used in a low-throughput drug screen. (a) Highly responsive cells chosen for the assay were expanded clonally, showing 70%–90% response to 25 mM butyrate (right), as compared to vehicle treated cells (left). (b) A 96-well plate used to screen compounds from the NCI structural diversity library was visualized after treatment with $4 \mu\text{M}$ compounds in DMSO, using a Discovery 1 fluorescent microscope platform and processed on the fly for 2×2 visualization per well. Hits are easily detectable by marked increase in fluorescence. Parental untransfected mouse mammary adenocarcinoma cells were used to counter screen.

TABLE 1: Hits from the screen are diverse in their structure. Butyrate was used as a positive control. The nonautofluorescent hits caused cells to express GFP, but have unique structures.

Name/ID	LDR Phenotype	Structure
Butyrate		
NSC-22225		
NSC-150117		
NSC-693322		
Secalonic Acid D; NSC-159631		

across all human cancer lines tested. To evaluate whether its ability to inhibit cell viability was specific for cancer versus normal cells, we measured its effects on a pair of matched lines derived from the lung cancer and the normal lung epithelium from the same patient. This revealed that secalonic acid D lacks specificity and inhibits a mechanism common to the survival of both normal and cancer cells (Figure 6(a)). This mechanism involves, at least in part, blocking of cell cycle progression in G2/M of the cell cycle, similar to the effects of TSA, as can be seen in Figure 6(b).

To directly test whether LDR hits had the ability to inhibit HDAC activity, we performed *in vitro* HDAC assays

in nuclear extracts, using a commercially available substrate conjugated to a fluorophore, which is cleaved only from deacetylated substrate in an activation step. Figure 6(c) demonstrates that at 10 μ M, only NSC-22206 inhibited HDAC activity. The lack of inhibition by NSC-22225 was surprising, leading us to confirm that the activity of NSC-22206 was specific and not the result of inhibition of the coupled activator reaction used in this assay. NSC-22206 did not affect the coupled reaction and was effective in inhibiting purified HDAC1 (Figure 6(c)) and in partly inhibiting HDAC8 (data not shown). In terms of the HDAC inhibition by NSC-22206, it must be concluded that its metal

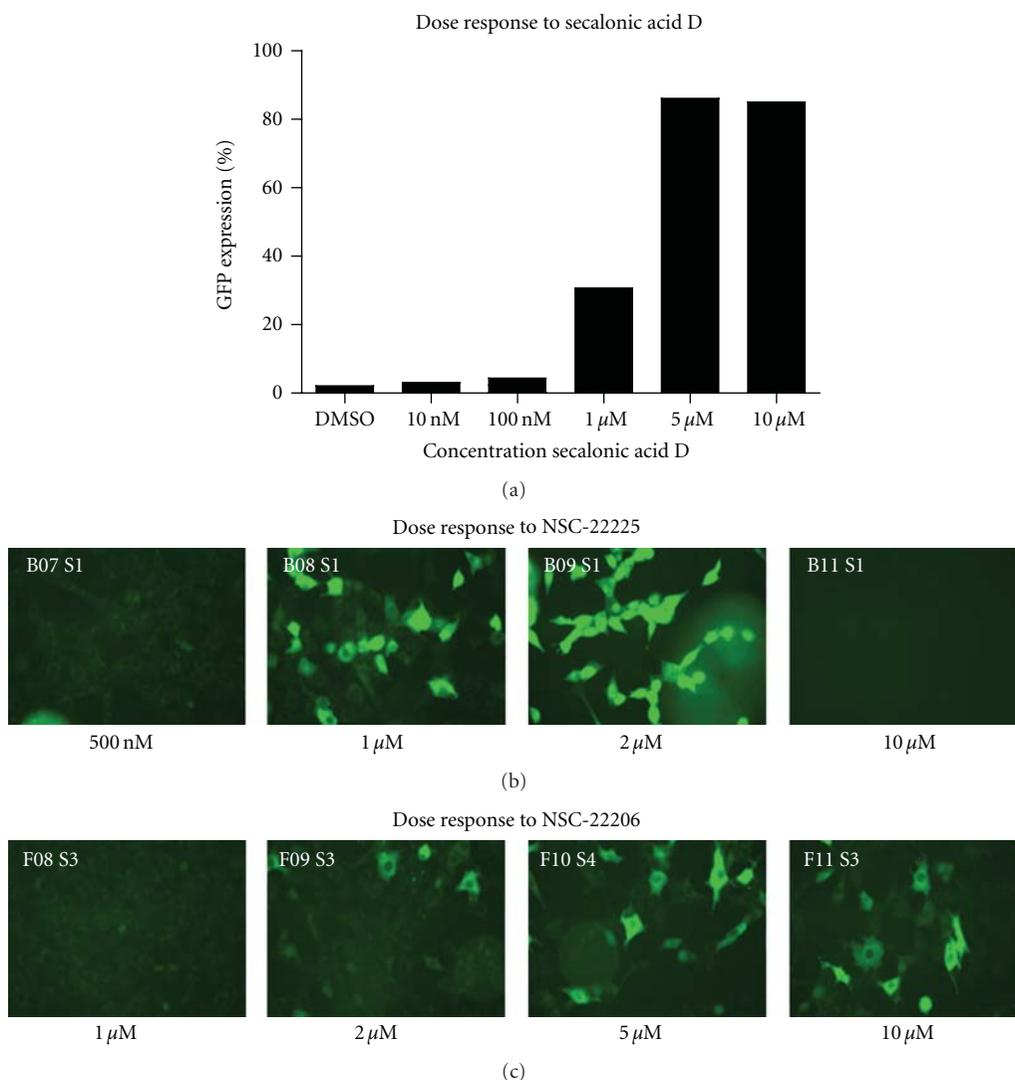


FIGURE 5: Characterization of assay hits. Dose-response experiments were performed with secalonic acid D, or NSC-159631 (a) and NSC-22225 (b), a related molecule NSC-22206 (c), which differs from NSC-22225 only by the metal salt (Cd for NSC-22225 and Hg for NSC-22206). In all three cases, GFP expression was dose dependent.

salt, mercury, but not the metal salt of NSC-22225, cadmium, contributes to the inhibition, potentially by replacing zinc in the HDAC catalytic site or by unspecifically binding cysteine thiols [35]. Indeed, 10 μ M mercury chloride on its own also inhibited HDAC activity *in vitro* although no GFP induction was seen in LDR cells (data not shown). To confirm the relevance of HDAC inhibition by NSC-22206 *in vivo*, we treated human lung cancer cells with this compound as well as with TSA as a positive control and secalonic acid as a negative control. Consistent with the HDAC activity data, cells treated with either 200 nM TSA or 10 μ M NSC-22206 showed increased levels of acetylated histone 3 and no changes in methylated histones (Figure 6(d) and Supplementary Figure 2B). In contrast, 0.5 μ M secalonic acid D, which inhibited cancer cell viability, had no effect on histone acetylation, yet surprisingly it did affect the global levels of histone 3 methylation in the same experiment

(Supplementary Figure 2B). This suggests that the ability of secalonic acid D to kill cells may be related to its cell cycle effects as mentioned above in combination with its global effects on histone methylation. In contrast, the effects of NSC-22206 on global histone acetylation are not sufficient to inhibit the viability of cancer cells. The possibility that in combination with other compounds our hits may be selective against cancer remains open to further investigation.

4. Conclusions

We have developed a cell-based system to identify compounds that potentially could affect a broad spectrum of epigenetic targets. Using this LDR assay to screen the NCI's structural diversity library, we have found four new epigenetic chemical modulators whose structure varies from known compounds in this class. Mechanistically, each small

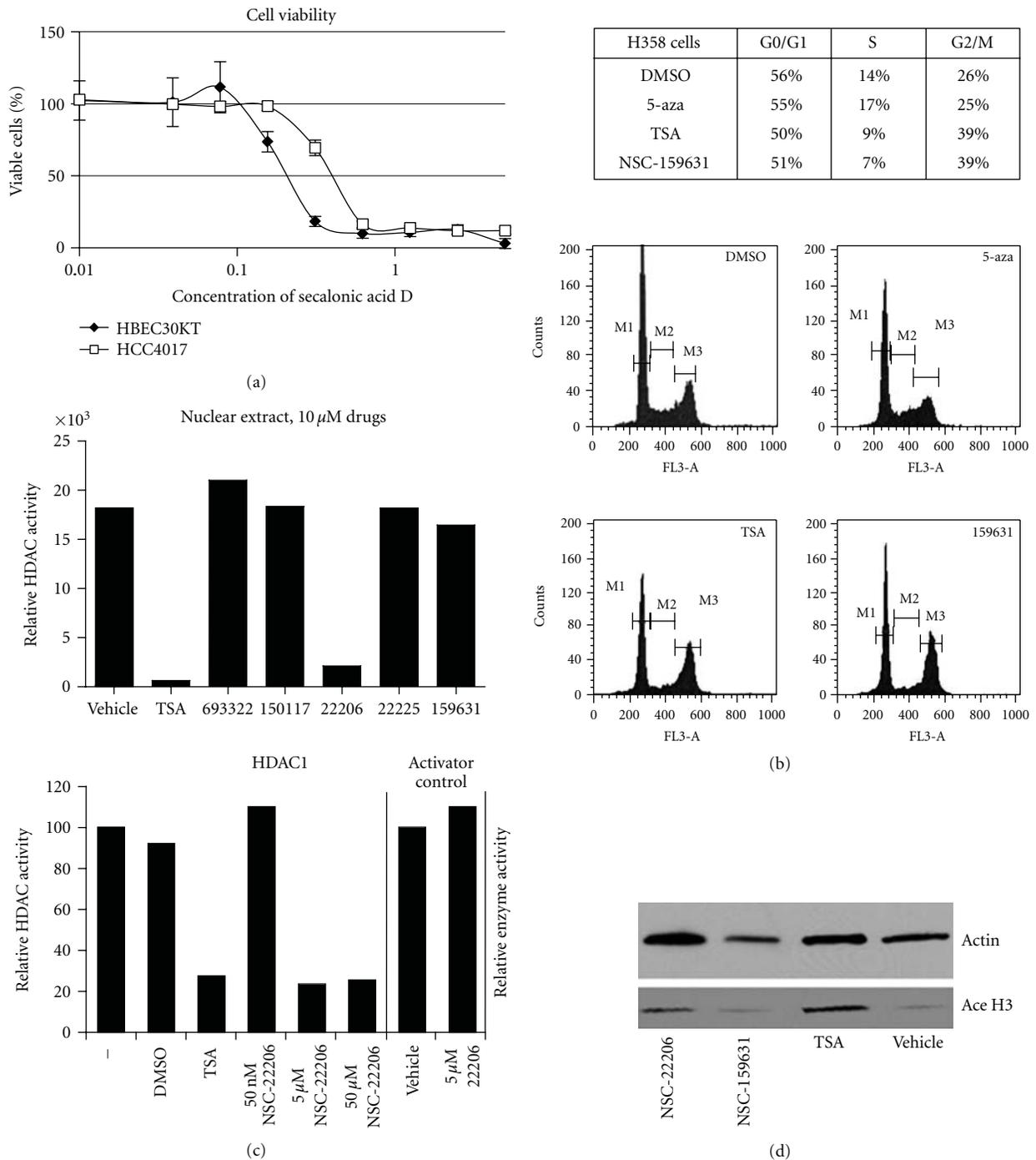


FIGURE 6: Characterization of LDR assay hits. (a) MTS Assay was performed with increasing doses of Secalonic acid D on a matched pair of lung cells: the lung cancer line HCC4017 and the normal line HBE30KT. Secalonic acid D did not show selectivity for cancer. (b) Cells were treated with vehicle or drug and sorted by cell-cycle phase after propidium iodide staining. TSA (200 nM) and NSC-15931 (5 μM) both caused cells to accumulate in G2/M phase as compared to vehicle or 5-azadeoxycytidine (1 μM). Values do not add to 100% because of the small fraction of sub G0/G1 cells. (c) *In vitro* HDAC activity assays were performed on nuclear extracts treated with 10uM hit compounds (left panel). Purified active HDAC1 activity was assayed in the presence of increasing concentrations of NSC-22206, which inhibited HDAC1 (right panel) but did not inhibit the coupled reaction (right side of panel). (d) H358 lung cancer cells were treated with drugs for 24 h and analyzed by Western Blot for changes in global histone modifications. NSC-22206 (10 μM) and TSA (200 nM) both caused an increase in levels of acetylated histone 3 whereas NSC-159631 (0.5 μM) did not.

TABLE 2: Characterization of the anticancer properties of hits. Cell viability assays were performed with hits from the assay, and IC₅₀ values were obtained for several nonsmall cell lung cancer cell lines and several melanoma cell lines (bold).

Drug ID	Cell Line	IC ₅₀ (μ M)
NSC-22225/NSC-22206	H1395	>20
	H1437	>10
	H358	>10
	Mel270	>5
	OCM3	>5
NSC-150117	H1395	2.3
	H1437	>10
	H358	1.9
	Mel270	1.7
	OCM1	3.4
	OCM3	2.2
NSC-693322	Omm1	0.5
	Omm2.3	1.7
	H1395	>10
	H1437	>10
	H358	8.7
	Mel270	1.5
	Mel285	1.7
	OCM1	1.4
OCM3	2.9	
NSC-159631	Omm1	0.1
	Omm2.3	0.3
	H1395	0.4
	H1437	0.7
	H358	0.6
	HCC4018	0.4
	Mel270	0.5
	Mel285	0.8
	OCM1	0.7
	OCM3	0.3
Omm2.3	0.5	

molecule hit exhibits unique properties in their ability to block cell cycle progression, inhibit HDACs, alter global histone acetylation, and induce cancer cell death. These chemical modulators may now be explored to define their specific molecular targets and to identify further their anticancer applications alone or in combination with existing anticancer drugs.

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Review Article

Beyond Histone and Deacetylase: An Overview of Cytoplasmic Histone Deacetylases and Their Nonhistone Substrates

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Acetylation of lysines is a prominent form of modification in mammalian proteins. Deacetylation of proteins is catalyzed by histone deacetylases, traditionally named after their role in histone deacetylation, transcriptional modulation, and epigenetic regulation. Despite the link between histone deacetylases and chromatin structure, some of the histone deacetylases reside in various compartments in the cytoplasm. Here, we review how these cytoplasmic histone deacetylases are regulated, the identification of nonhistone substrates, and the functional implications of their nondeacetylase enzymatic activities.

1. Introduction

Acetylation is one of the most prominent posttranslational modifications affecting the functions of proteins. The first acetylated protein identified is histone [1]. Twenty-seven years after this discovery, histone acetyltransferases (HATs) and histone deacetylases (HDACs) were identified to be the enzymes responsible for the acetylation and deacetylation of lysines of histone proteins [2, 3]. A year later, lysine modification of nonhistone proteins was confirmed with p53 [4]. Shortly after, many nonhistone proteins were found to be subject to acetylation/deacetylation (reviewed in [5]). Remarkably, a recent genome-wide screening for acetylated proteins shows that lysine acetylation of nonhistone proteins regulates the formation of large protein complexes with key roles in major cellular processes [6]. Therefore, lysine acetylation, both in histones and nonhistone proteins, appears to be a salient mode of regulation in the cell.

In mammals, there are eleven protein deacetylases bearing the names HDACs and seven SIRT proteins homologous to yeast Sir2. The subcellular distribution of these HDACs is not limited to the nucleus. Some of them, like HDAC6 and HDAC10, are distributed mainly in the cytoplasm, while SIRT3, 4, and 5 are mitochondrial proteins. A few of them, including HDAC3, SIRT1, and SIRT2, shuttle between the

nucleus and the cytoplasm. In this paper, we choose to focus on nonhistone substrates of HDACs, including those in the nucleus and those in the cytoplasm. We will also discuss how these deacetylases are regulated and any non-deacetylase functions they might possess.

2. HDAC3

Subcellular localization distinguishes HDAC3 from other class I HDAC subfamily members. HDAC3 shuttles between the nucleus and the cytoplasm by a CRM1-mediated pathway [7, 8], suggesting that dynamic localization is a key regulator of the function of HDAC3. In addition to targeting histones during interphase (reviewed in [9]), HDAC3 also targets substrates in the cytoplasm and during mitosis, when the nuclear envelope breaks down and the distinction between nucleus and cytoplasm becomes blurred. Table 1 summarizes the subcellular localization and the functions of nonhistone substrates of HDAC3.

2.1. Nonhistone, Nuclear Substrates of HDAC3

2.1.1. MEF2. MEF2 regulates myogenesis and other developmental processes, including apoptosis and cardiac hypertrophy. It has long been shown to interact with class II HDACs

such as HDAC4 and HDAC5, but how it is deacetylated was unclear. It was later found that MEF2 is sumoylated, not deacetylated, by HDAC4 (discussed in Section 3.4). Interestingly, the classical HDAC that deacetylates MEF2 was identified as HDAC3, and HDAC3 deacetylates MEF2 to repress the MyoD promoter [10].

2.1.2. Export of SRY and PCAF to the Cytoplasm. HDAC3 affects transcription through a two-fold mechanism: by deacetylating targets in the nucleus and by promoting their nuclear exclusion as a result of deacetylation. Deacetylation of SRY by HDAC3 delocalizes SRY to the cytoplasm [11]. Moreover, HDAC3 promotes translocation of PCAF to the cytoplasm during apoptosis [12]. Coexpression HDAC3 with PCAF not only promotes cytoplasmic retention of PCAF but also that of HDAC3, suggesting a novel interplay between HDAC3 and PCAF. HDAC3-mediated deacetylation might reveal nuclear export signals of substrate proteins and help with their interaction with the CRM1-importin machinery [7].

2.2. Cytoplasmic Substrates of HDAC3. HDAC3-mediated deacetylation of STAT1 prevents its phosphorylation, which is required for nuclear translocation and DNA binding of STAT1 [13]. In addition, HDAC3 activity also regulates STAT3 phosphorylation. HDAC3 deacetylates STAT3 at lysine 685, and an acetylation-resistant mutant of STAT3 loses the dimerization ability [14]. HDAC3 was also found to form complexes with STAT3 and PP2A to mediate dephosphorylation of STAT3 [15]. These findings imply that HDAC3 might be responsible for the cytoplasmic retention of STAT3 as well as STAT1. However, factors or signals that activate HDAC3 remain elusive.

2.3. NF- κ B: A Special Substrate of HDAC3 Traversing the Nucleus and the Cytoplasm. HDAC3 deacetylates p65 [16], one of the two components of the most abundant form of the NF- κ B heterodimer. Deacetylation of p65 at lysine 221 promotes binding of p65 with I κ B and rapid nuclear exportation. Deacetylation of lysine 310, however, impairs the transcriptional activity of p65 without changing its DNA-binding activity or nuclear export [17]. Acetylation of p65 was also found on lysines 122/123. Deacetylation of these two lysines by HDAC3 maintains p65 in an active state in the nucleus [18]. Interestingly, HDAC3 itself associates with I κ B in the cytoplasm but translocates into the nucleus under TNF- α -induced I κ B degradation [19]. These findings suggest that HDAC3 differentially regulates the activity of NF- κ B through deacetylating different lysine residues of p65 and is regulated by I κ B for cytoplasmic retention.

2.4. Regulation of HDAC3 by Phosphorylation. HDAC3 was found in a protein complex with PP4, and it can be phosphorylated by CK2 [20]. Biochemically, phosphorylation decreases the enzymatic activity of HDAC3 [20]. PP2A also forms a complex with HDAC3 [15]. HDAC3 is phosphorylated at multiple serine/threonine residues during differentiation of endothelial progenitors and interacts with

Akt. This interaction promotes phosphorylation of Akt, increasing its kinase activity [21]. However, despite the apparent phosphorylation of HDAC3, whether phosphorylation changes its subcellular localization, substrate specificity, or deacetylase activity in the cytoplasm remains unclear. More systematic studies are needed to clarify how and why HDAC3 is regulated by phosphorylation in a physiological context.

2.5. Association of HDAC3 with the Mitotic Chromosome. HDAC3 was first found to associate with the mitotic chromosome through immunoaffinity purification of the Flag-HDAC3 complex, in which two mitotic chromosome-associated proteins AKAP95 and HA95 were found [22]. Knockdown of HDAC3 by siRNA blocks histone H3 deacetylation and H3S10 phosphorylation, suggesting H3 modification by this complex. However, the role of AKA95 and HA95, most likely in the recruitment of HDAC3 to the mitotic chromosome, remains unclear. Detailed studies on the role of HDAC3 in mitosis were further extended with add-back strategies using wild-type or deacetylase-dead mutants in HDAC3-knockdown cells. HDAC3 and its enzyme activity are required for the formation of functional mitotic spindles and proper kinetochore-microtubule attachment [23]. Fine mapping on chromosomes, using the same strategies, demonstrates that H3K4 deacetylation by HDAC3 is required for centromeric functions during mitosis. Loss of HDAC3 impairs centromeric H3T3 phosphorylation, Sgo1 localization, and H3K4 methylation during mitosis, leading to abnormal sister-chromatid separation and impaired centromeric functions in mitosis [24]. These findings support a novel, nontranscriptional function of HDAC3 in the cytoplasm during mitosis. It will be interesting to know if AKA95, HA95, and Sgo1 are also substrates of HDAC3. Further identification of nonhistone targets associated with the mitotic chromosome might reveal more functional links between HDAC3 and mitosis.

2.6. Non Deacetylase Function of HDAC3. A recent report about how phosphorylated orphan nuclear receptor TR2 moves to PML nuclear bodies demonstrated that nuclear relocation of TR2 by HDAC3 is deacetylase independent using TSA and HDAC3 deacetylase-dead mutants [25]. This finding suggests a novel function of HDAC3 as a molecular chaperone and alludes to a previous report that HDAC3 requires proper folding and priming by a chaperone complex called TRiC (TCP-1 ring complex) [26]. Geldanamycin, an inhibitor of chaperone functions, inhibits the formation of the TRiC-HDAC3 complex [26]. Interestingly, a recent report showed that TRiC complexes are acetylated [6]. It remains to be uncovered if the histone deacetylase enzyme activity of HDAC3 affects the chaperone function of TRiC and if HDAC3 has innate chaperone activity.

3. HDAC4, 5, 7, 9

HDAC4, 5, 7, and 9 are classified into the class IIa subfamily of HDACs due to similarities in primary structure. Indeed,

TABLE 1: Subcellular localization and functions of nonhistone substrates of HDAC3.

Substrates	Localization of substrates	Functions	Comments
MEF2	Nucleus	Repression of MyoD promoter	HDAC3 is the only Class I HDAC that regulates MEF2 activity
SRY	Nucleus	Nuclear export	Deacetylation leads to cytoplasmic delocalization Cytoplasmic translocation promotes apoptosis
PCAF	Nucleus	Cytoplasmic retention	Functional interplay between PCAF and HDAC3
STAT1	Cytoplasm	Prevention of STAT1 phosphorylation	
STAT3	Cytoplasm	Prevention of dimerization	
NF- κ B	Cytoplasm or nucleus	Cytoplasmic retention or activation of p65 in the nucleus	Functions are dependent on the acetylation lysines

they share similar functions in deacetylating histones in the nucleus and in phosphorylation-regulated subcellular localization (reviewed in [27, 28]). Recent progress on HDAC4 and 7 provides us with valuable information on new substrates, mode of regulation, and novel enzymatic functions of these HDACs. Table 2 summarizes the subcellular localization and the functions of nonhistone substrates of HDAC4 and HDAC7.

3.1. Nonhistone, Nuclear Substrates of HDAC4, and HDAC7.

The first reported nonhistone protein substrates of HDAC4 are p53 and Runx2. HDAC4 deacetylates lysines at the C-terminus of p53, resulting in an increase in the transcriptional repression activity of p53 under DNA damage [29]. Acetylation of Runx2 was found during BMP-2-stimulated osteoblast differentiation and bone formation. HDAC4 mediates deacetylation of Runx2 and promotes Smurf-mediated degradation of Runx2 [30].

It is reported that HDAC7 shuttles into the nucleus under hypoxia and functions as a co-repressor for HIF1 α [31]. Although HDAC7 interacts with HIF1 α and increases the transcriptional activity of HIF1 α , whether HDAC7 deacetylates HIF1 α remains to be determined.

3.2. Cytoplasmic Substrates of HDAC4.

Recently three proteins were found to be potential targets for deacetylation by HDAC4. The Z-disc-associated protein, MLP, can be acetylated by PCAF and deacetylated by HDAC4 in the cytoplasm [32], which is important in the regulation of muscle contraction. HIF1 α interacts with HDAC4 and the cytoplasmic localization of HDAC4 is required for the activity of HIF1 α [33]. As HIF1 α is destabilized by acetylation [34], HDAC4 might help with deacetylation of HIF1 α in the cytoplasm and maintenance of neuronal survival. Moreover, DNAJB8, which is a cytoplasmic chaperone important for suppressing cytotoxic protein aggregation, interacts with HDAC4. Inhibition of HDAC4 reduces the function of DNAJB8 [35]. More cytoplasmic substrates might be found for this subfamily of HDACs in the future.

3.3. Regulation of Nucleocytoplasmic Shuttling of HDAC4 and HDAC7.

Nucleocytoplasmic shuttling of HDAC4 is controlled by phosphorylation and dephosphorylation (reviewed in [28, 36]). Phosphorylated HDAC4 binds to 14-3-3 and stays in the cytoplasm. This cytoplasmic retention might be due to preferential binding of phosphorylated HDAC4 with 14-3-3 in the cytoplasm, resulting in a decrease in nuclear import [37]. Nuclear import of HDAC4 can be attained by dephosphorylation of serine 298 by PP2A, possibly changing the conformation to expose the nuclear localization signal (NLS) [38]. HDAC4 can also be imported into the nucleus through interacting with MEF2C by using MEF2C's NLS [38, 39]. A recent paper found that HDAC4-interacting protein DNAJB5 is required for its nuclear retention as loss of this interaction relocates HDAC4 to the cytoplasm [40].

In addition to phosphorylation, oxidation also regulates nucleo-cytoplasmic shuttling of HDAC4. Oxidation of HDAC4 at cysteines 667/669 results in the formation of intramolecular disulfide bonds in response to cardiac hypertrophy [40]. In this report, overexpression of a newly identified HDAC4-interacting protein, Trx1 (Thioredoxin 1), suppresses nuclear export of HDAC4 in response to reactive oxygen species (ROS) induced by phenylephrine [40]. It seems that Trx1 regulates the localization of HDAC4 independently of phosphorylation, suggesting multiple ways to regulate nuclear retention of HDAC4.

Finally, proteolysis might control nuclear import of HDAC4 [41, 42]. HDAC4 is cleaved at aspartate 289 by caspase processing, leaving behind the C-terminus of HDAC4 in the cytoplasm [42]. The N-terminal portion of HDAC4 possesses an NLS and accumulates in the nucleus. This fragment also represses the transcriptional activity of MEF2C and increases apoptosis [41, 42]. Figure 1 summarizes how nucleo-cytoplasmic shuttling of HDAC4 is regulated.

HDAC7 shuttles into the nucleus under hypoxia through an unknown mechanism [43]. It also specifically localizes to PML NBs through interacting with PML [44, 45]. Ectopic expression of PML relieves the repressive effect of HDAC7 on

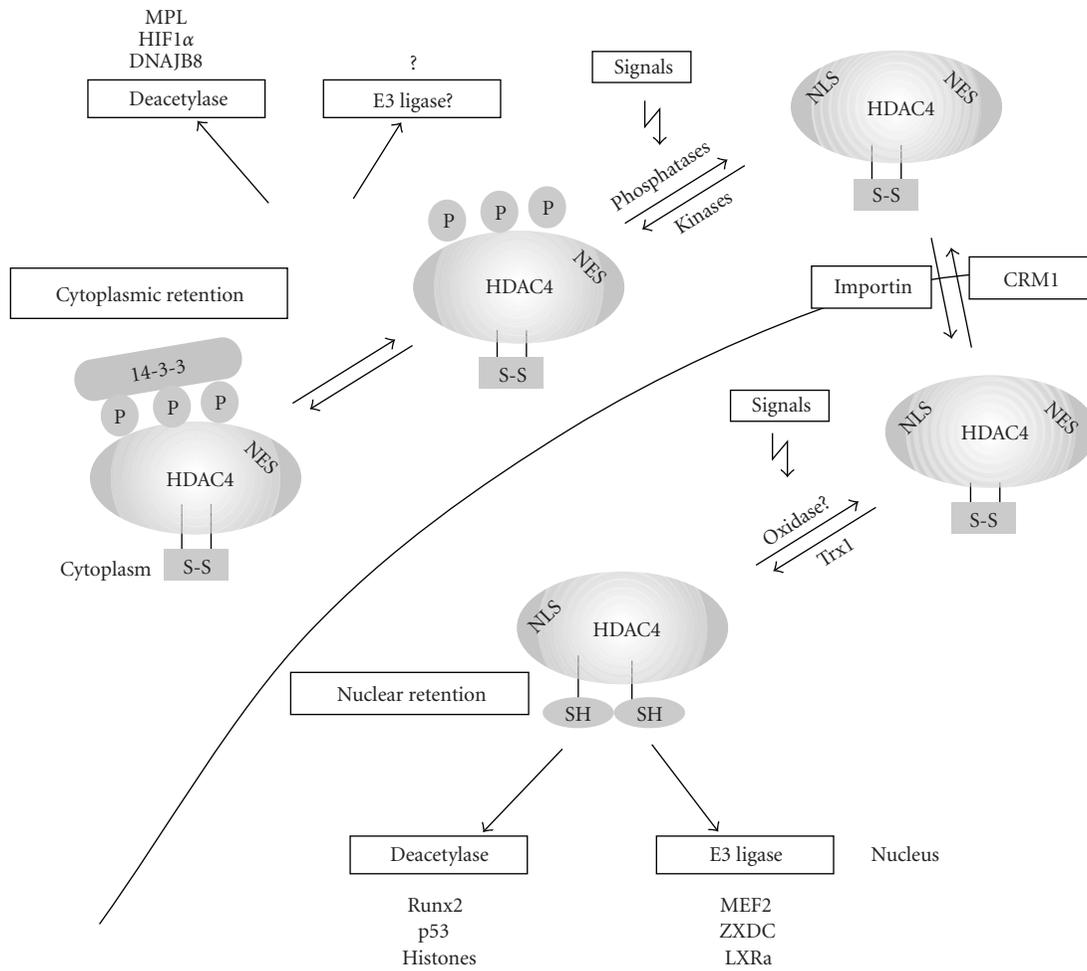


FIGURE 1: A model on the regulation of nucleo-cytoplasmic shuttling and the functions of HDAC4. HDAC4 dynamically shuttles between nucleus and cytoplasm, depending on its phosphorylation status. Phosphorylated HDAC4 binds to 14-3-3 and remains in the cytoplasm. The cytoplasmic form of HDAC4 might possess protein deacetylase activity. Whether cytoplasmic HDAC4 possesses SUMO E3 ligase activity remains unclear. Dephosphorylated HDAC4 is imported into the nucleus, where reduction of HDAC4 by Trx1 favors nuclear retention. Oxidases that catalyze the reverse reaction remain to be identified. Nuclear HDAC4 possesses deacetylase as well as SUMO E3 ligase activities on substrates indicated in the figure.

TABLE 2: Subcellular localization and functions of nonhistone substrates of HDAC4 and HDAC7.

Histone deacetylases	Substrates	Localization of substrates	Functions	Comments
HDAC4	p53	Nucleus	Increases repressional activity of p53	Under DNA damage conditions
	Runx2	Nucleus	Promotes Smurf-mediated degradation of Runx2	
	MPL	Cytoplasm	Regulation of muscle contraction in cardiac mechanical stretch	
	HIF1 α	Cytoplasm	Cytoplasmic retention to maintain neuronal survival	
	DNAJB8	Cytoplasm	Suppression of cytotoxic protein aggregation	
HDAC7	HIF1 α	Nucleus	Corepressor for HIF1 α	Deacetylation is not confirmed for this interaction

androgen receptor-mediated transcription by sequestering HDAC7 into PML NBs [44, 45].

3.4. Sumoylation: A Non-deacetylase Function of HDAC4 and HDAC7. HDAC4 was found to be sumoylated at lysine 559 [46]. When this lysine is mutated to arginine, HDAC4 loses its transcriptional repression and histone deacetylase activity [46]. Interestingly, HDAC4 is also a SUMO E3 ligase *in vivo* and *in vitro*, targeting MEF2 at lysines 439 and 424 [47, 48]. Sumoylation of MEF2 by HDAC4 potentiates the transcriptional repression activity of MEF2 specifically for muscle differentiation genes [48]. As MEF2 lysine 424 is acetylated by CBP, these findings suggest that the transcriptional activity of MEF2 is regulated by a balance between acetylation and sumoylation, and sumoylation by HDAC4 may prevent lysine acetylation. Recently, additional sumoylation substrates have been attributed to HDAC4. Sumoylation of a transcription factor called ZXDC by HDAC4 enhances its transcriptional activity [49]. LXR is sumoylated with SUMO2/3 by HDAC4, which turns LXR into a transcriptional co-repressor regulating programs of gene expression that control immunity and homeostasis [50]. LXR α , but not LXR β , specifically associates with the STAT1/HDAC4 complex, whereas HDAC4 promotes sumoylation of LXR α . Furthermore, sumoylation of LXR α is required for the suppression of STAT1-dependent inflammatory responses by LXRs in brain astrocytes stimulated with IFN- γ [51].

HDAC7 was reported to facilitate transcriptional repression in a deacetylase-independent manner [52], which might be a result of sumoylation of HDAC7-interacting proteins. HDAC7 associates with PML, promoting sumoylation of PML as well as the formation of PML NBs [53]. It is possible that the putative E3 enzyme activity of HDAC7 is important for the regulation of androgen receptor-mediated transcription (compare Section 3.3).

4. HDAC6 and 10

HDAC6 is a cytoplasmic, class IIb HDAC. Most of the studies focus on its substrate tubulin and how (de)acetylation of tubulin affects lymphocyte chemotaxis, cellular adhesions, aggresome formation, EGFR signaling, HIV infection, stress granules in stress response, and growth factor-induced actin remodeling and endocytosis (reviewed in [54–58]). Here, we focus on recent identification of novel HDAC6 substrates, regulation of HDAC6 deacetylase activity through protein-protein interactions (summarized in Figure 2 and Table 3), and the biological function of a close relative of HDAC6, HDAC10.

4.1. Cytoplasmic Substrates of HDAC6

4.1.1. Hsp90. Hsp90 was the second HDAC6 substrate identified in the cytoplasm after tubulin. Although Hsp90 can be acetylated at different lysines, HDAC6 specifically deacetylates lysine 294 of Hsp90 [64]. Deacetylation of this lysine decreases the chaperone function of Hsp90, specifically Hsp90's interaction with cochaperone p23 and client protein glucocorticoid receptor (GR) ([65]; reviewed in [54]).

Deacetylation of Hsp90 by HDAC6 results in retention of p23 and GR in the cytoplasm, failure of GR maturation, and decreased transcription of target genes. This model is further examined in aryl hydrocarbon receptor (AhR) signaling. Inhibition of HDAC6 blocks the formation of the chaperone complex containing Hsp90, AhR, p23, and XAP-2 as well as the activation of downstream target genes [66].

4.1.2. Cortactin. HDAC6 deacetylates cortactin, an F-actin-binding protein. Deacetylation of cortactin increases its interaction with F-actin [67]. A mutation of cortactin that mimics acetylation prevents its localization to membrane ruffles and inhibits cell motility. HDAC6 knockdown causes the same effect [67]. Based on these findings, Lee and his colleagues found that deacetylation of cortactin by HDAC6 is important for autophagy ([68]; reviewed in [69]).

4.1.3. β -Catenin. β -catenin is found to be acetylated at lysine 49 [70], which is frequently mutated in anaplastic thyroid cancer. Acetylation of lysine 49 blocks phosphorylation of serine 45 of β -catenin [70], which promotes its degradation [71, 72]. Therefore, HDAC6 inactivation inhibits EGF-induced nuclear translocation β -catenin, resulting in inhibition of cellular growth [70]. This finding suggests a new role of HDAC6 in the regulation of specific signal transduction pathways.

4.1.4. Peroxiredoxins. Peroxiredoxin (Prx) I and Prx II are antioxidants that reduce H₂O₂, a process important in the modulation of intracellular redox status. Deacetylation of Prx I and Prx II by HDAC6 leads to apoptosis [73], suggesting that HDAC6 regulates stress response by altering redox homeostasis.

4.2. Regulation of HDAC6 by Protein-Protein Interaction.

Unlike class IIa HDAC members, HDAC6 has not been shown to be regulated through nucleo-cytoplasmic shuttling. Interestingly, several recent reports describe that HDAC6 enzymatic activity is negatively regulated by protein-protein interaction partners (Figure 2 and Table 3), [59–63]. For example, interaction with Iip45 renders HDAC6 unstable [61], while EGFR signaling results in HDAC6 phosphorylation at tyrosine 570, reducing its deacetylase activity [63]. Studies from these newly identified HDAC6-interacting proteins suggest that, in addition to direct chemical inhibition, HDAC6 can be subject to regulation by extracellular and intracellular stimuli.

4.3. HDAC10: An Enigmatic Relative of HDAC6. While HDAC6 plays multiple roles in the cytoplasm, very little is known about another cytoplasmic deacetylase, HDAC10, which is closest to HDAC6 (reviewed in [55]). Recently, HDAC10 is shown to target nonhistone substrates both in the nucleus and the cytoplasm [74]. Using immunoaffinity purification, Hsp70 was identified as the most abundant protein associated with HDAC10 and HDAC10 only interacts with a deacetylated form of Hsp70, suggesting that HDAC10 might contribute to the deacetylation status of Hsp70 [74].

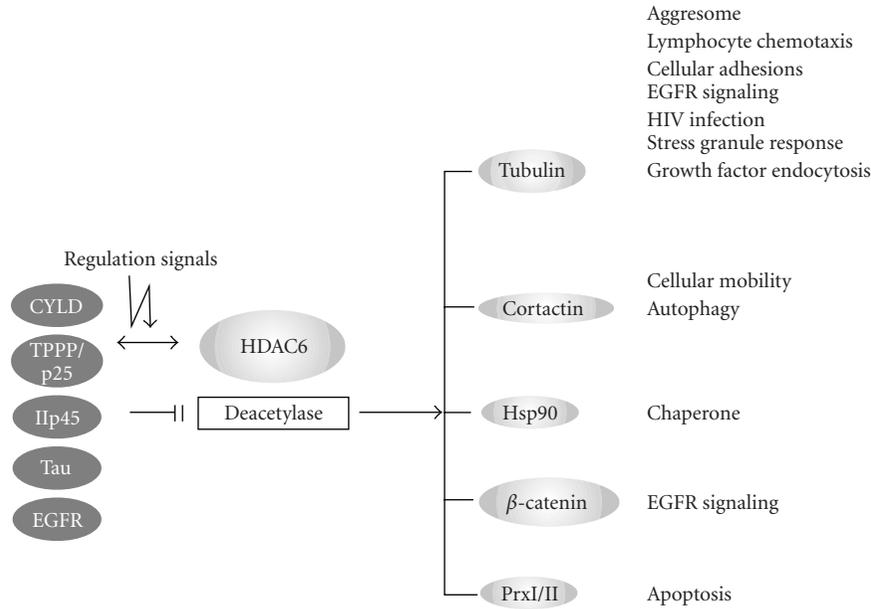


FIGURE 2: Regulation and functional ramifications of the HDAC6 deacetylase activity. Interaction with protein partners reduces protein deacetylase activity of HDAC6, which is important for the deacetylation of substrate proteins and cellular effects as indicated.

TABLE 3: Proteins interacting with and inhibiting the deacetylase activity of HDAC6.

Interacting proteins	Substrates affected	Functions involved	References
TPPP/p25	Tubulin	Decrease cell mobility	[59]
CYLD	Tubulin	Cell proliferation	[60]
Iip45	Tubulin (decreases stability of HDAC6)	Cell migration	[61]
Tau	Tubulin	Prevent autophagy	[62]
EGFR	Tubulin (decreases deacetylase activity of HDAC6 by phosphorylation)	Decrease endocytosis and degradation	[63]

Using a specific histone deacetylase inhibitor FK228 to treat K562 cells, acetylation of Hsp70 is increased while that of Hsp90 remains unchanged [75]. Interestingly, acetylated Hsp70 specifically interacts with c-Abl and Bcr-Abl in K562 cells, promoting Bcr-Abl degradation [75]. These results suggest that acetylation of Hsp70, which possibly results from deregulation of HDAC10 rather than HDAC6, might cause incorrect folding of substrate proteins in the cytoplasm and subsequent degradation (Figure 3). Furthermore, HDAC10 might target nonhistone proteins important in transcriptional regulation. HDAC10 interacts with deacetylated forms of Pax3 and KAP1, resulting in derepression of Pax3 target genes during melanogenesis [74]. These lines of evidence suggest that, like HDAC6, HDAC10 has nonhistone targets in both the nucleus and the cytoplasm. Full revelations of the physiologic functions of HDAC10 will come from identification of additional HDAC10 targets.

5. SIRT1–5

Sir2 is an NAD⁺-dependent deacetylase that extends life span in yeast. Mammalian SIRT proteins, or sirtuins, are

yeast Sir2 homologs that regulate diverse processes including life span, energy metabolism, stress response, DNA repair, and tumorigenesis. Seven mammalian SIRT proteins have been identified to date. Most reviews focus on SIRT1, the closest relative of yeast Sir2, and SIRT2 (for a very recent review on SIRT1 and 2, see [76]). Mitochondrial sirtuins are usually reviewed separately [77–79]. In this section, we will briefly discuss the functions of SIRT1 and SIRT2 in the cytoplasm as their general (nuclear) functions are extensively reviewed in the literature, summarize the functions of SIRT3, 4, 5 that reside in the mitochondrion, and omit chromatin-associated SIRT6 and nucleolar-residing SIRT7. For a concise overview of the function of all seven sirtuins, readers are referred to recent reviews such as [80–82]. Table 4 summarizes the substrates and the functions of these cytoplasmic sirtuins.

5.1. SIRT1. The subcellular localization of SIRT1 is cell type dependent. Studies using cell lines and animals show that in some cells SIRT1 is predominately nuclear while in others it is predominately cytoplasmic [83, 84]. Two nuclear localization signals and two nuclear export signals have been identified in SIRT1 [83, 84]. Cytoplasmic localization of SIRT1, mediated

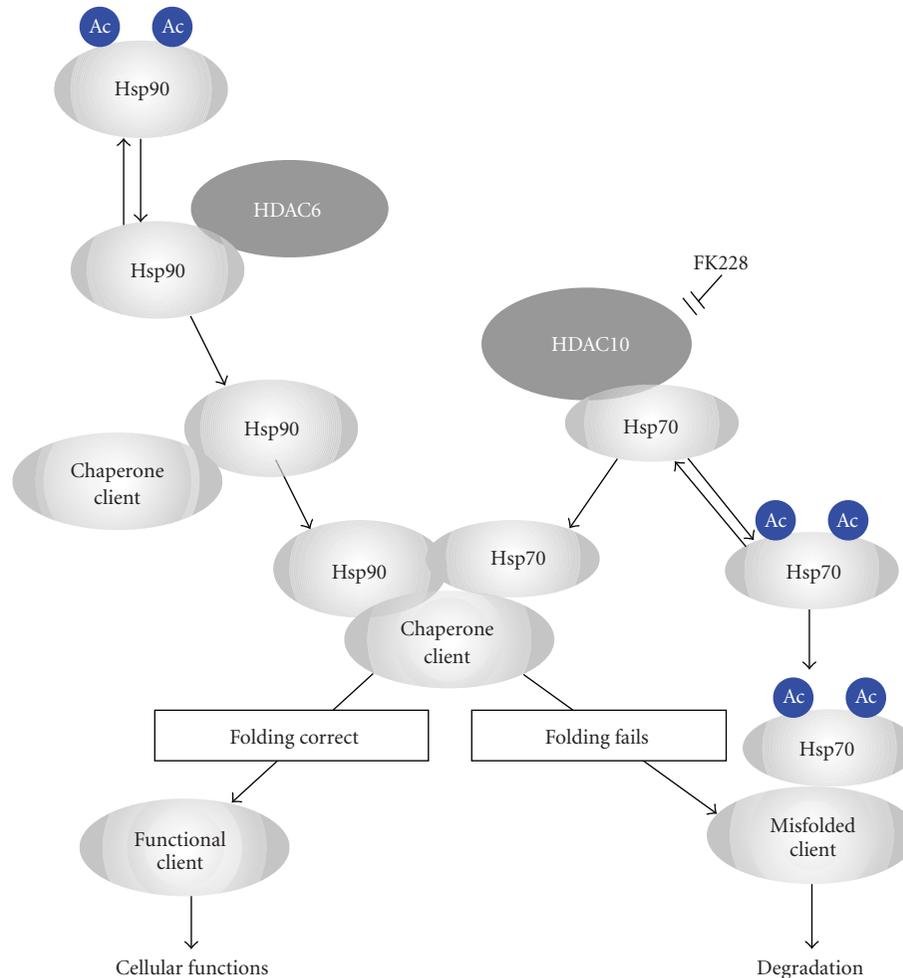


FIGURE 3: A model of how HDAC6/Hsp90 and HDAC10/Hsp70 collaboratively work as protein chaperones. Deacetylation of Hsp90 catalyzed by HDAC6 might prime its chaperone function. Deacetylated Hsp70, catalyzed by HDAC10 or by another deacetylase, joins the Hsp90/chaperone client complexes to help with correct folding of the clients. Acetylation of Hsp70, a likely result of HDAC10 deregulation, might cause incorrect folding of proteins or facilitate the subsequent degradation of misfolded proteins.

by a CRM1-dependent nuclear export pathway, might be regulated by differentiation [83]. However, the cytoplasmic presence of SIRT1 has also been linked to a propensity for apoptosis, as cytoplasmic translocation of nuclear SIRT1 increases apoptosis [83, 84].

Questions remain about the physiologic significance of cytoplasmic SIRT1 as well as the detailed mechanism of apoptotic regulation by cytoplasmic translocation of SIRT1. It has been shown, almost a decade ago, that SIRT1 suppresses stress-induced apoptosis by deacetylating p53 [85, 86]. In studies using mammalian cells and tissues, cytoplasmic SIRT1 promotes apoptosis induced by oxidative stress and nocodazole, a metaphase apoptosis inducer [83, 84]. These results suggest that elucidation of how SIRT1 responds to cellular stress and then translocates to the cytoplasm will be key to full understanding of how SIRT1 regulates apoptosis.

In genetic assays using *C. elegans*, it appears that SIR-2.1, the worm homolog of SIRT1, also induces apoptosis in a manner that depends on its cytoplasmic translocation from

the nucleus [87]. However, this study shows that SIR-2.1 specifically activates the apoptotic program induced by DNA damage in germ cells [87]. It will be interesting to find out whether the differences in the worm study and the studies in mammalian cells are a result of experimental design, or SIRT1-mediated apoptosis indeed has inherent specificity.

5.2. SIRT2. SIRT2 is predominately cytoplasmic [88], with nuclear-cytoplasmic shuttling reported [89, 90]. The biological function of SIRT2 was first revealed by the identification that SIRT2 is an NAD^+ -dependent deacetylase specific for α -tubulin [88]. A number of nuclear substrates deacetylated by SIRT2 has also been identified, including p53, FOXO1, FOXO3a, histone H4, histone H3, and p300 [91–96], whereas the biological significance of these nuclear substrates will be excluded from the discussion here. The deacetylase activity of SIRT2 is modulated by phosphorylation and acetylation, although the exact subcellular localization of these modifications remains unspecified. Phosphorylation of the SIRT2 protein at serine 368, which inhibits SIRT2

TABLE 4: Cytoplasmic sirtuins, their substrates in the cytoplasm, and their functions.

Mammalian cytoplasmic sirtuins	Substrates	Functions	Comments
SIRT1 (subcellular localization is cell type dependent)		Cytoplasmic localization of SIRT1 is linked to apoptosis	Whether SIRT1 enhances or decreases apoptosis and the mechanistic details remain unclear
SIRT2 (shuttles between nucleus and cytoplasm)	α -tubulin	Mitotic exit of normal cell cycle Regulation of mitotic checkpoint under stress Neuronal motility and differentiation	Subcellular localization of SIRT2 modifications that modulate deacetylase activity of SIRT2, namely, phosphorylation and acetylation, is largely unspecified Mechanism(s) through which SIRT2 regulates mitotic exit remains unclear
SIRT3 (in mitochondria)	AceCS2 GDH ICDH2 Complex I of electron transport chain Ku70 Nampt MRPL10	Regulation of energy metabolism and apoptosis	GDH activity is not significantly altered in <i>Sirt3</i> knockout mice
SIRT4 (in mitochondria)	GDH (SIRT4 also interacts with ANT2, SIRT3, and IDE)	Regulation of insulin secretion	SIRT4 is an ADP ribosylase No deacetylase activity is reported for SIRT4 ADP-ribosylation of ANT2, SIRT3, or IDE by SIRT4 has not been shown definitely
SIRT5 (in mitochondria)	CPS1 cytochrome <i>c</i>	Regulation of urea cycle Promotes apoptosis?	Exact submitochondrial localization of SIRT5, how calorie restriction alters CPS1 acetylation through SIRT5, and what lysines in CPS1 are acetylated under different nutrient availability remain controversial

activity, is mediated by cyclin-dependent kinases Cdk1, Cdk2, and Cdk5 [97, 98]. Dephosphorylation of SIRT2 is attributed to CDC14B [97, 99]. Acetylation of SIRT2 by p300, which is also a substrate of SIRT2, downregulates the activity of SIRT2 [100]. SIRT2 also interacts with HDAC6, HOXA10, 14-3-3 β/γ , and members of the DNAJB chaperone family [35, 88, 92, 101].

Compared with other mammalian sirtuins, SIRT2 expresses abundantly in the brain [98, 102]. Given that SIRT2 deacetylates α -tubulin, SIRT2 might be instrumental in the regulation of neuronal processes involving microtubules, such as neurite outgrowth, dynamics of cytoskeletal growth cones, oligodendrocyte arborization, and migration of glioma (reviewed in [103]). SIRT2 has also been implicated in the proliferation of gliomas [104]. Further studies show that SIRT2 regulates mitotic exit from the normal cell cycle as well as mitotic checkpoints under mitotic stress induced by microtubule inhibitors ([99, 105, 106] and reviewed in [82]).

SIRT2 has been implicated in the regulation of mitotic exit. It accumulates during G2/M transition, and over-expressed SIRT2 prolongs mitosis [99, 101]. Furthermore, microinjection of O-acetyl-ADP-ribose formed during SIRT2-mediated deacetylation or low amounts of human

SIRT2 blocks starfish oocyte maturation [107]. It is postulated that a decrease in the amount or enzymatic activity of SIRT2 is required for proper mitotic exit. Findings showing how SIRT2 is regulated by cyclin-dependent kinases and CDC14B, which is important for cytokinesis, seem to support this hypothesis. However, the detailed mechanism of how SIRT2 regulates mitotic exit remains elusive.

Studies from the Oshimura group further found that SIRT2 blocks chromosome condensation in the presence of microtubule poisons such as nocodazole, enabling a mitotic checkpoint [105, 106], a phenomenon previously ascribed to the prophase checkpoint protein CHFR [108]. SIRT2 appears to be necessary to rescue cells from prolonged mitotic arrest and to avoid secondary outcomes such as cell death and hyperploidy that usually accompany release from such mitotic arrest [106]. Furthermore, downregulation of SIRT2 using siRNA causes centrosome fragmentation before the activation of the mitotic checkpoint elicited by nocodazole [106]. These results suggest that SIRT2 is crucial in the regulation of mitotic checkpoints and centrosome integrity.

5.3. *SIRT3*. SIRT3 is a mitochondrial matrix protein that regulates mitochondrial protein acetylation [109–111], and

its N-terminal mitochondrial targeting sequence needs to be removed for full deacetylase activity [111, 112]. It has been suggested that SIRT3 might translocate to the nucleus [113–115], and the nuclear presence of SIRT3 requires both the mitochondrial targeting signal as well as the putative nuclear localization signal [113]. The physiological significance of the presence of SIRT3 in the nucleus, the mitochondrion, or even the cytoplasm remains controversial (reviewed in [116]). Some studies suggest that it depends on whether the SIRT3 protein is proteolytically processed [115], as both a long form and a short form of the SIRT3 protein have been reported [117–120] and cleavage of SIRT3 affects subcellular location [115].

The substrates of SIRT3 known to date fall into two categories: those involved in energy metabolism and those involved in apoptosis. The former group includes acetyl-coenzyme A synthase 2 (AceCS2) [111, 121], glutamate dehydrogenase (GDH) [79], isocitrate dehydrogenase 2 (ICDH2) [79], and Complex I of the electron transport chain [122]. The latter includes Ku70 [115], nicotinamide phosphoribosyltransferase (Nampt) [123], and MRPL10 (mitochondrial ribosomal protein L10) [124].

Deacetylation of AceCS2 at lysine 642 by SIRT3 activates the enzymatic activity of AceCS2 [111, 121], which converts acetate into acetyl-CoA [125]. GDH converts glutamate to α -ketoglutarate [126]. In vitro deacetylation of GDH by SIRT3 activates its activity by 10% [79], and GDH is hyperacetylated in *Sirt3* knockout mice [109]. However, GDH activity is not significantly altered in *Sirt3* knockout mice [127]. SIRT3 deacetylates and activates the activity of ICDH2 in vitro in a dose-dependent manner [79]. Components of the electron transport chain Complex I show increased acetylation levels and decreased activity in *Sirt3*^{-/-} cells [122]. SIRT3 also physically interacts with the NDUFA9 subunit of Complex I [122].

In cardiomyocytes, SIRT3 deacetylates Ku70 and promotes association between Ku70 and Bax, thereby preventing Bax-mediated apoptosis [115]. Studies using siRNA show that SIRT3 (and SIRT4) is required for Nampt-mediated protection against methylmethane sulfonate-induced cell death [123]. Nampt catalyzes the rate-limiting step in NAD⁺ biosynthesis from NAM [128, 129] and is upregulated by fasting or cell stress [123]. Upregulation (an increase in protein amount) of Nampt by nutrient deprivation might be a result of SIRT3-mediated deacetylation and activation of mitochondrial ribosomal protein MRPL10, which enhances mitochondrial protein synthesis [124]. Interestingly, angiotensin II (Ang II) downregulates the mRNA level of *Sirt3*, and this downregulation is inhibited by application of an antagonist to Ang II type 1 receptor [130]. These results suggest that SIRT3 promotes longevity and cellular response to different forms of stress through a multiple of mechanisms. However, as SIRT3 is also reported as a proapoptotic factor [131], the role of SIRT3 in apoptosis requires additional evidence to clarify.

5.4. SIRT4. SIRT4 resides in the mitochondrial matrix [132–134]. SIRT4 has not been shown to possess protein deacetylase activity. Instead, it ADP ribosylates glutamate

dehydrogenase (GDH) [133]. In mitochondria, GDH catalyzes the conversion of glutamate to α -ketoglutarate, which subsequently enters the tricarboxylic acid cycle [126]. ADP ribosylation by SIRT4 inhibits the enzymatic activity of GDH and hinders the metabolism of glutamine and glutamate to generate ATP [133]. As a result, SIRT4 represses insulin secretion of pancreatic β cells in response to glucose and amino acids.

Further evidence that SIRT4 regulates insulin secretion comes from the finding that SIRT4 associates with adenine nucleotide translocator 2 (ANT2) [132]. Metabolites from glycolysis and tricarboxylic acid cycle fuel the production of ATP through oxidative phosphorylation, which leads to an increase in the level of ATP in the mitochondrial matrix. ATP/ADP translocators such as ANT2 facilitate exchange of mitochondrial ATP for cytosolic ADP, causing an increase of the cytosolic ATP/ADP ratio. In response, ATP-sensitive K⁺ channels close, which leads to plasma membrane depolarization and opening of the voltage-dependent Ca²⁺ channels. The resulting flux of intracellular Ca²⁺ prompts insulin exocytosis (reviewed in [135]). Therefore, findings that SIRT4 ADP ribosylates GDH and interacts with ANT2 strongly support a role of SIRT4 in the regulation of insulin secretion. An inverse relationship between GDH activity and ADP-ribosylation is also observed during calorie restriction (CR) in that hepatic GDH activity is elevated while the level of ADP-ribosylation of GDH is decreased [133]. Interestingly, CR induces amino acid-stimulated insulin secretion in pancreatic islets [133, 136]. These phenomena allude to previous findings that, during CR, amino acids serve as carbon sources [136, 137]. However, they do not explain the need to upregulate the activity of GDH and the secretion of insulin during CR.

The studies surrounding GDH and insulin secretion inevitably raise the question of how the ADP-ribosylase activity of SIRT4 is regulated. Unfortunately, there is no clear answer to that question. However, SIRT3, another mitochondrial sirtuin that interacts with Complex I of the electron transport chain [122] and shows weak deacetylase activity toward GDH [79], interacts with SIRT4 [132]. SIRT4 also interacts with insulin-degrading enzyme (IDE), although it has not been shown clearly whether IDE can be ADP-ribosylated by SIRT4 [132]. It is tempting to speculate that the activity of SIRT4 is regulated by another sirtuin, perhaps by NAD⁺-dependent deacetylation or by a certain kind of insulin-sensing mechanism. Further experimental support is needed to answer these questions.

5.5. SIRT5. The N-terminal 36 amino acids of SIRT5 are removed after SIRT5 is imported into the mitochondrial matrix [127, 138]. However, debates remain surrounding the exact location of SIRT5 in the mitochondrion. Endogenous murine SIRT5 shows exclusive matrix localization [127]. However, overexpressed SIRT5 is located in the mitochondrial intermembrane space (IMS) in COS7 cells [113]. To add to the confusion, in vitro import assay using isolated yeast mitochondria shows that human SIRT5 is imported into both the IMS and the matrix [79]. There is also a report showing that SIRT5 exists in both the nucleus and

the cytoplasm [139]. To fully understand the physiological significance as well as regulation of SIRT5, the exact cellular location of this sirtuin needs to be determined.

The *in vivo* substrate of SIRT5 has recently been identified as carbamoyl phosphate synthase 1 (CPS1) in an immunocapture screen for SIRT5-interacting partners [127]. CPS1 is the committed enzyme of the urea cycle, which is important for the removal of excess ammonia [140, 141]. Excess ammonia is generated during fasting or as a result of high-protein diet [141–144]. Activation of CPS1 by SIRT5 is, therefore, crucial for the conversion of ammonia into carbamoyl phosphate, and ultimately, urea. NAD⁺-dependent deacetylation of CPS1 by SIRT5 activates the enzymatic activity of CPS1 *in vitro*, and CPS1 activity is decreased in *Sirt5* knockout mice [127]. Nutrient deprivation induces CPS1 activity in normal primary hepatocytes [127]. More importantly, during starvation, *Sirt5* knockout mice show higher levels of blood ammonia, lower CPS1 activity, and higher levels of CPS1 acetylation compared with wild-type animals [127]. However, in *Sirt5*^{-/-} cells, CPS1 activity is less sensitive to nutrient deprivation treatment [127].

It appears that CR for 6 months brings about a decreased level of CPS1 acetylation in normal mice, lower than that observed for animals fed *ad libitum* but higher than in fasted animals [127]. However, the acetylation level of CPS1 has also been reported to be increased by CR [145]. Potential controversy exists regarding the level and particular lysines acetylated in CPS1 in response to varied nutrient availability (discussed in [78]). Along the lines of energy metabolism, it has been shown that the mitochondrial level of NAD⁺ increases about twofold while that of NADH remains constant during fasting, and SIRT5 activity is stimulated by NAD⁺ with a K_m of 50–100 μ M *in vitro* [127]. Moreover, ethanol causes a decrease in both NAD⁺ level [146] and the activity of SIRT5 [147]. These findings raise the possibility that SIRT5 is regulated by the level of NAD⁺. However, as NAD⁺ affects the activity of many sirtuins, more biochemical analysis is needed to discriminate the differences between sirtuins in their response to NAD⁺ levels.

In addition to CPS1, cytochrome *c* has been reported to be deacetylated by SIRT5 in an ELISA-based system and colocalizes with SIRT5 in the IMS [79]. It is possible that SIRT5-mediated deacetylation contributes to the regulation of the proapoptogenic activity of cytochrome *c*. Furthermore, overexpressed and mitochondrially localized SIRT5 promotes apoptosis in cerebellar granule neurons and in HT-22 neuroblastoma cells [139]. The role of SIRT5 in apoptosis no doubt depends on its exact subcellular location, and the causal relationship between SIRT5 and apoptosis remains to be clarified.

6. Concluding Remarks

Acetylation is a rather common form of post-translational modification [6]. However, when, how, and which deacetylases regulate the acetylation status of proteins remain largely unknown. Recent studies about HDAC3 and NF- κ B also remind us that deacetylation of different lysines on the same substrate protein can have divergent consequences.

Therefore, the field of protein acetylation is far from mature. Future advances will likely come from understanding of how the activity of deacetylases is regulated as well as identification of novel, non-deacetylase activities. We now know that multiple inputs, coming from interactions with cytoplasmic proteins, are integrated into HDAC6 to regulate its deacetylase activity. Moreover, HDAC4 and HDAC7 participate in protein sumoylation, while HDAC3 might function as a protein chaperone. Similar discoveries will further enlighten us on this fascinating group of proteins and clarify some of the controversies surrounding the mechanistic details of mammalian sirtuins.

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Review Article

Jun Dimerization Protein 2 Controls Senescence and Differentiation via Regulating Histone Modification

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Transcription factor, Jun dimerization protein 2 (JDP2), binds directly to histones and DNAs and then inhibits the p300-mediated acetylation both of core histones and of reconstituted nucleosomes that contain JDP2 recognition DNA sequences. JDP2 plays a key role as a repressor of adipocyte differentiation by regulation of the expression of the gene *C/EBP δ* via inhibition of histone acetylation. Moreover, JDP2-deficient mouse embryonic fibroblasts (JDP2^{-/-} MEFs) are resistant to replicative senescence. JDP2 inhibits the recruitment of polycomb repressive complexes (PRC1 and PRC2) to the promoter of the gene encoding p16^{Ink4a}, resulting from the inhibition of methylation of lysine 27 of histone H3 (H3K27). Therefore, it seems that chromatin-remodeling factors, including the PRC complex controlled by JDP2, may be important players in the senescence program. The novel mechanisms that underline the action of JDP2 in inducing cellular senescence and suppressing adipocyte differentiation are reviewed.

1. Introduction

The structure of chromatin, which influences numerous DNA-associated phenomena, such as transcription, replication, recombination, and repair, is controlled by a complex combination of histone modifications, ATP-dependent chromatin-remodeling enzymes, and nucleosome-assembly

factors [1, 2]. The modification of histones such as acetylation, phosphorylation, methylation, ubiquitination, sumoylation, and ADP-ribosylation can regulate the gene expression [1–4]. The chromatin consists of structural units known as nucleosomes. Each nucleosome consists of two histone H2A-H2B dimers, a histone (H3-H4)₂ tetramer, and DNA that is wrapped around the resultant histone octamers.

During chromatin assembly, a histone (H3-H4)₂ tetramer is formed before the two heterodimers of histones H2A and H2B are incorporated to form a nucleosome [5, 6]. The regulation of transcription is associated with alterations in chromatin structure that include histone modifications and changes in nucleosome structure [7–10]. Compaction of the chromatin and organization of nucleosomes represent a barrier that has to be overcome prior to the activation of transcription. The N-terminal histone tails that protrude from nucleosomes do not play a significant role in nucleosome formation but, rather, they appear to act as docking sites for other proteins and protein complexes to regulate chromatin compaction [9].

The structure of chromatin changes to allow greater accessibility by transcription factors when a gene is to be activated [10]. It has been suggested that the change to a more accessible state not only involves the modification of histones and alterations in nucleosomal arrays but also results from changes in nucleosome integrity that are due to displacement of histones [11]. Furthermore, it has been demonstrated that histone chaperones play a critical role in these processes [12–15]. Thus, it is tempting to speculate that histone chaperones might be important for the compaction of chromatin, and it is now important to determine whether certain corepressors of transcription might influence the deposition and assembly of nucleosomes through the regulation of histone-chaperone activity. The transcription factor Jun dimerization protein 2 (JDP2) is a member of AP-1 family that binds to both AP-1 site and cAMP responsive element (CRE) site in various *cis*-elements of the target genes. It is generally accepted that the transcription factors have DNA-binding activities and then control their transcriptional activities by DNA binding. However, we found that JDP2 has not only DNA-binding activity but also histone-binding activity. Moreover, JDP2 bound the nucleosome in both DNA sequence-dependent or -independent manner. JDP2 also has the nucleosome assembly activities and the activities of inhibition of histone acetyltransferase and histone methyltransferase. Here, we describe the role of JDP2 on the adipocyte differentiation and replicative senescence in mouse embryonic fibroblasts through histone modification.

2. JDP2 Regulates the AP-1-Mediated Activation of Transcription

JDP2 has been identified as a binding partner of c-Jun in yeast two-hybrid screening experiments, based on the recruitment of the SOS system [16]. JDP2 forms heterodimers with c-Jun and represses the AP-1-mediated activation of transcription [16]. Similarly, JDP2 was isolated by yeast two-hybrid screening with activation transcription factor-2 (ATF-2) as the “bait” [17]. JDP2 was also shown to associate with both the CCAT/enhancer-binding protein gamma (C/EBPγ) [18] and the progesterone receptor [19]. JDP2 is expressed in many cell lines and represses the transcriptional activity of AP-1 [20]. Moreover, JDP2 is rapidly phosphorylated at threonine residue 148 when cells are exposed to UV irradiation, oxidative stress, or

inhibitor-induced depressed levels of translation by c-Jun NH(2)-terminal kinase (JNK) [21]. Although a novel JNK-docking domain is necessary for the activated kinase (MAPK) p38-mediated phosphorylation of JDP2 at threonine residue 148, this domain is not sufficient for this process [22]. JDP2 binds to both cAMP-responsive element (CRE) and TPA-responsive elements (TREs) on DNA as a homodimer and as a heterodimer with ATF-2 and members of the Jun family, respectively [16, 17]. JDP2 encoded an 18 kDa protein that is able to homodimerize as well as to form heterodimers with other AP-1 members, such as c-Jun, JunB, JunD, and ATF-2, and members of the C/EBP family, C/EBPγ and C/EBP homologous protein 10 (CHOP 10) [16, 17] as well as Interferon regulatory factor-1 (IRF-1) binding protein 1 [23]. Dimerization occurs through a conserved leucine zipper domain found all members of the AP-1 family. A basic domain located adjacent to the leucine zipper dimerization motif is responsible for the direct association with TRE and CRE [16, 17]. *In vitro* study using the purified JDP2 protein showed that JDP2 forms the homotrimer but not the homodimer in our biochemical condition (unpublished data). JDP2 inhibits UV-induced apoptosis by suppressing the transcription of the p53 gene [24]. Given the roles of AP-1 in cellular transformation and the reported repression of Jun- and ATF-2-mediated transcription by JDP2, we have demonstrated that JDP2 inhibits the oncogenic transformation of chicken embryonic fibroblasts [25]. JDP2 also modulates the expression of cyclin D1 and p21, which have opposing effects on cell-cycle progression. JDP2 interferes with the progression of the cell cycle by reducing the levels of cyclin D1 and at the same time increases the expression of p21 [26, 27]. The forced expression of JDP2 promotes the myogenic differentiation of C2C12 cells, which is accompanied by the formation of C2 myotubes and the strong expression of major myogenic markers. Moreover, the ectopic expression of JDP2 in rhabdomyosarcoma cells induces incomplete myogenesis and the incomplete formation of myotubes [27]. These cells become committed to differentiation via the p38-MAPK pathway [21, 22]. A similar enhancement of cell differentiation was reported during the induction of osteoclast formation by the receptor activator of the nuclear factor κB (NFκB) ligand (RANKL) [28]. Unlike other members of the AP-1 family, the levels of JDP2 remain constant in response to a large variety of stimuli, such as UV, irradiation, and retinoic acid (RA), which affect the levels of other factors involved in cell-cycle control. The induction of JDP2 expression was only observed during the differentiation of F9 cells to muscle cells and osteoclasts. Therefore, JDP2 may provide a threshold for exit from the cell cycle and a commitment to differentiation (Figure 1). Further studies of the regulation of the cell cycle and the differentiation of cells induced by JDP2 should be very instructive. It is also interesting that JDP2 is one of the candidate oncoproteins that collaborate in the oncogenesis associated with the loss of p27 as the result of insertional mutations [29]. Recent study of tumor cells demonstrated that JDP2 was a tumor suppressor [30]. We have also found that JDP2 is a repressor of the activation of transcription via AP-1 and a negative regulator of the retinoic acid- (RA-)

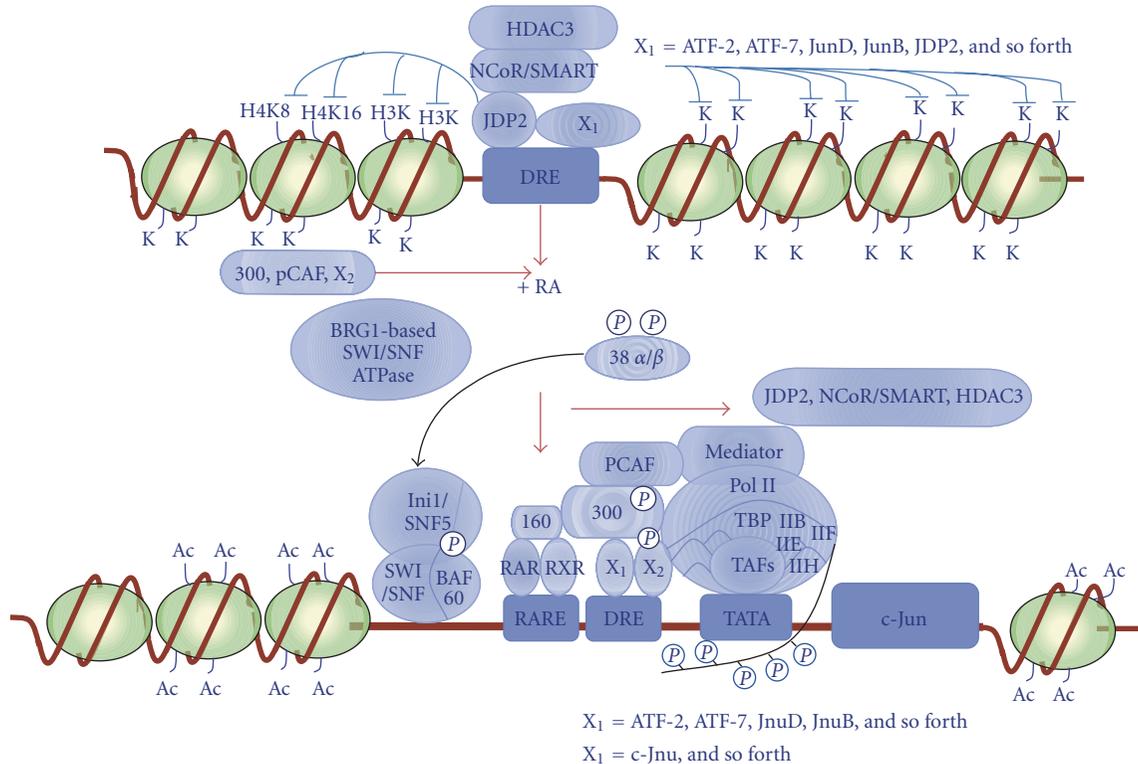


FIGURE 1: Schematic representation of the signal pathways of retinoic acid- (RA-) induced (RA-induced) differentiation of mouse embryonic carcinoma F9 cells. At the undifferentiation stage of F9 cells, HDAC3, NcoR/SMART, and JDP2 were recruited on the DRE (differentiation response element) in the promoter region of the *c-jun* gene to induce the heterochromatin. In the response to retinoic acid (RA), the signals of mitogen-activated kinase (MAPK; phosphorylated (p38(α/β))), BRG1-based SWI/SNF ATPase complex, Ini1/Snf5/SWI/SNF/BAF60 complex, p160 hormone coactivator (RARE/RXRE binding), and p300/PCAF complex were recruited to the DRE element of the *c-jun* promoter and then Mediator, Pol II complex (IIB, IIF, IIE, IIF complex), TBP complex, and some TAF complex (ATF12, TAF 4 etc), and recruited and Pol II complex is elongated with phosphorylation of the carboxy terminal domain (CTD) of Pol II, and then *c-Jun* genes are finally activated. Ac, acetylated residues; K, lysine residues of histone; H3K, lysine residues of histone H3; H4K8, lysine residue at position 8 of histone H4; H4K16, lysine residue at position 16 of histone H4; $X_1 = \text{ATF-2, ATF-7, JunD, JunB, JDP2, and so forth}$; $X_2 = \text{c-Jun and so forth}$.

induced differentiation of mouse embryonic F9 cells and adipocyte differentiation [31–33].

3. JDP2 Inhibits Histone Acetyltransferase (HAT) Activity

We have reported previously that JDP2 represses the transactivation mediated by p300 [32]. Both p300 and ATF-2 have HAT activity [34, 35]. It was recently shown that p300 acetylates ATF-2 protein *in vitro* at lysine residues 357 and 374 and that ATF-2 is essential for the acetylation of histones H4 and H2B *in vivo* [36, 37]. We found that acetylation by p300 is inhibited in a dose-dependent manner by JDP2, when added exogenously. We also found that JDP2 was not acetylated by p300 under our experimental conditions. The inhibitory effect of JDP2 was detected on histone acetylation induced by p300, CREB-binding protein (CBP), p300/CBP-associated protein (PCAF), and general control nonrepressive 5 (GCN5). The overexpression of JDP2 apparently represses the RA-induced acetylation of lysines

8 and 16 of histone H4 and some amino terminal lysine residues of histone H3.

4. JDP2 Has Intrinsic Nucleosome-Assembly Activity *In Vitro*

The template activation factor-1 β protein (TAF-1 β protein), which is a component of the inhibition of histone acetylase complex (INHAT complex) identified by Seo et al. [38, 39], is a histone chaperone that binds directly to core histones and facilitates the assembly of nucleosomes *in vitro*. JDP2 interacted directly with all the core histones tested and inhibited the p300-mediated acetylation of those histones. To our surprise, JDP2 also introduced supercoils into circular DNA in the presence of core histones, to levels similar to those observed for yeast CCG1-interacting factor 1 protein (γ Cia1p) and CCG1-interacting factor (CIA1). Therefore, JDP2 appears to have significant histone chaperone activity *in vitro* [32]. We have also shown that the HAT-inhibitory activity of JDP2 is involved, to some extent, in the repression of transcription by JDP2, whereas the maximal capacity

of JDP2 to suppress the RA-mediated activation of the *c-Jun* promoter [32, Figure 1] and to suppress the adipocyte differentiation [33] requires the recruitment of histone deacetylases (HDACs).

5. JDP2 Suppresses Adipocyte Differentiation

JDP2 has been shown to play a role in the cellular differentiation of skeletal muscle, osteoclasts, adipocytes [27, 33], and F9 cells [31]. It has been reported that JDP2 has an activity of tumor suppressor using prostate cancer cells [30]. Recently, we also found that JDP2-deficient mouse fibroblasts are resistant to replicative senescence [40]. Finally general inhibition of the AP-1 complex by expression of JDP2 specifically in the heart correlates with the induction of atrial dilatation [41]. In mammals, the strict control of adipocyte development, the mass of adipose tissue, the insulin sensitivity of adipocytes, and the appropriate metabolism of glucose and lipids are critical to the maintenance of energy homeostasis [42, 43]. Adipogenesis, namely, the process whereby hormonal stimuli induce the differentiation of fibroblasts or mesenchymal cells to adipocytes, requires the organized and controlled expression of a cascade of transcription factors and the modification of the chromatin within preadipocytes [44–47]. The factors involved in adipocyte differentiation include a nuclear receptor known as peroxisome proliferation-activated receptor gamma (PPAR γ) and a group of C/EBPs [48, 49]. The rapid and transient induction of the expression of C/EBP β and C/EBP δ is one of the earliest events in adipogenesis [46]. These transcription factors bind to specific sequences in the promoters of the C/EBP α gene and the PPAR γ gene, inducing their expression, which, in turn, activates the full adipogenic program of gene expression [50–53]. Expression of PPAR γ is also induced via a sterol-regulating element-binding protein-1c- (SREBP-1c-) dependent pathway [54]. Once both PPAR γ and C/EBP α are activated, “cross-talk” between PPAR γ and C/EBP α maintains the expression of each protein during adipocyte differentiation, even in the absence of C/EBP β and C/EBP δ [55]. We generated JDP2 “knock-out” (KO) mice in order to study the activities of JDP2 in vivo, and we found that JDP2 plays a role as a repressor of adipocyte differentiation. We also found that JDP2 targeted an adipogenesis-related gene, C/EBP δ , and inhibited its expression via regulation of histone acetylation.

The C/EBP δ and C/EBP α mRNAs were overexpressed in *Jdp2*^{-/-} MEFs during the initial stages of adipocyte differentiation, whereas C/EBP β and PPAR γ mRNAs were less affected by the absence of JDP2 [56]. C/EBP α is known to be the downstream target of C/EBP δ and C/EBP β [57]; therefore, it can be speculated that the augmentation of expression of the C/EBP gene might have been due to indirect effects, which were probably caused by the enhanced expression of the C/EBP δ gene [33]. In fact, the recruitment of JDP2 to the C/EBP δ and C/EBP β genes was detected, but there was no obvious recruitment to the C/EBP α gene. Thus, the target of JDP2 is possibly the C/EBP δ and C/EBP β genes.

JDP2 was able to bind to the C/EBP δ promoter to repress the transcription of the C/EBP δ gene. Our JDP2-deficient

mice did not have abundant adipose cells, in other words, they did not have a thick layer of fat tissue, perhaps because the mass of adipose tissue might be determined by more complex factors, such as cytokines and hormones, via as yet unknown mechanisms, and by energy status. We found that the adipose tissue of scapulae from young *Jdp2*^{-/-} mice consisted mostly of white adipocytes, whereas the majority of cells were brown adipocytes in WT mice. This observation suggested that JDP2 might play a role in adipogenesis *in vivo*.

Recently, it has been reported that the activation of Wnt signalling inhibited the development of brown adipocytes by mediating the peroxisome proliferation-activated receptor gamma-1 coactivator-1-1 α - (PGC1-1 α -) uncoupling protein1 (UCP1) cascade [48]. In addition to C/EBPs as shown in the present studies, JDP2 might be a target of such signal networks. Other report suggested that the absence of Rb switched the differentiation of mouse embryonic fibroblast (MEF) cells from white to brown adipocytes [58], suggesting that the differentiation of WT MEFs to adipocytes is a good model for white-adipocyte differentiation but not for brown-adipocyte differentiation. We also observed that PGC-1 α marker of brown adipocyte was not induced after induction of adipocyte differentiation in *Jdp2*^{-/-} MEFs and WT MEFs (unpublished). Then, we analyzed the levels of mRNA of PGC-1 α in Adeno-JDP2-infected 3T3-L1 cells. 3T3-L1 cells are also known to be a model for white-adipocyte differentiation, however, we could detect small amount of PGC-1 α RNA by real-time RT-PCR as the others reported elsewhere [59] probably because quite minor population of 3T3-L1 cells can differentiate to brown adipocyte. The expression of PGC-1 α was increased after adipocyte differentiation, and the levels of expression were not remarkably different between Adeno-JDP2- and Adeno-lacZ -infected 3T3-L1 cells. So, we speculate that JDP2 might inhibit the differentiation of white adipocytes exclusively, but not brown adipocytes. In consequence, brown adipocytes are preferentially developed in WT mice scapulae, whereas white adipocytes appear in *Jdp2*^{-/-} mice. However, further studies are required to clarify the role of JDP2 in the molecular mechanism that directs fibroblast and mesenchymal cells to differentiate into white or brown adipocytes.

6. JDP2 Controls Replicative Senescence

We analyzed the aging-dependent proliferation of MEFs from *Jdp2*^{-/-} mice in the presence of environmental (20%) or low (3%) oxygen [40]. The *Jdp2*^{-/-} MEFs continued to divide, even after six weeks, whereas the wild-type MEFs almost stopped proliferating and entered senescence under environmental oxygen. Conversely, neither the wild-type MEFs nor the *Jdp2*^{-/-} MEFs succumbed to replicative senescence at lower oxidative stress. These results demonstrate that MEFs lacking JDP2 can escape from the irreversible growth arrest caused by environmental oxygen. The expressions of p16^{Ink4a} and alternative reading frame (Arf) were repressed in aged *Jdp2*^{-/-} MEFs (40 days) compared with their levels in wild-type MEFs. In 3% oxygen, at the equivalent time (40 days), wild-type MEFs expressed lower levels of p16^{Ink4a} and

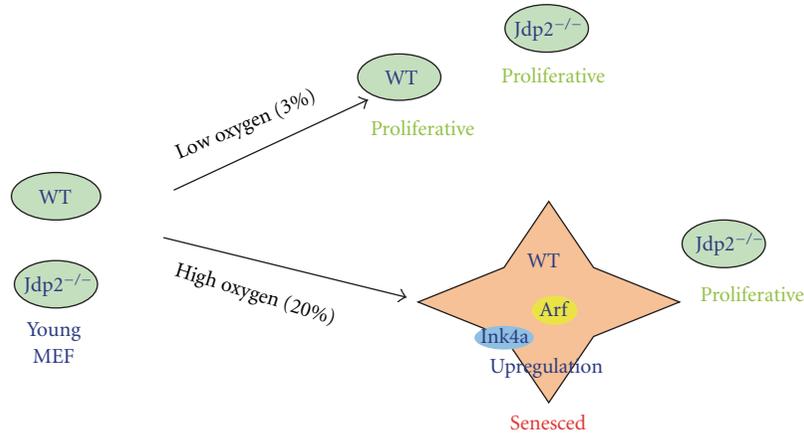


FIGURE 2: Oxidative stress controls the cell proliferation of mouse MEF cells through the epigenetic regulation of the $p16^{Ink4a}/Arf$ locus by JDP2. Young MEF primary cells exposed to oxidative stress (20%) accumulate JDP2. In the presence of JDP2, PRC1, and PRC2 dissociate from the $p16^{Ink4a}/Arf$ locus, and histone H3 on the promoter is demethylated. Finally, $p16^{Ink4a}$ and Arf are upregulated to express and then aged cells senescence. In the absence of JDP2, MEF cells do not enter the senescence stage and proliferate well. In the lower oxygen (3%), both WT and $Jdp2^{-/-}$ MEF cells are not senesced but proliferated.

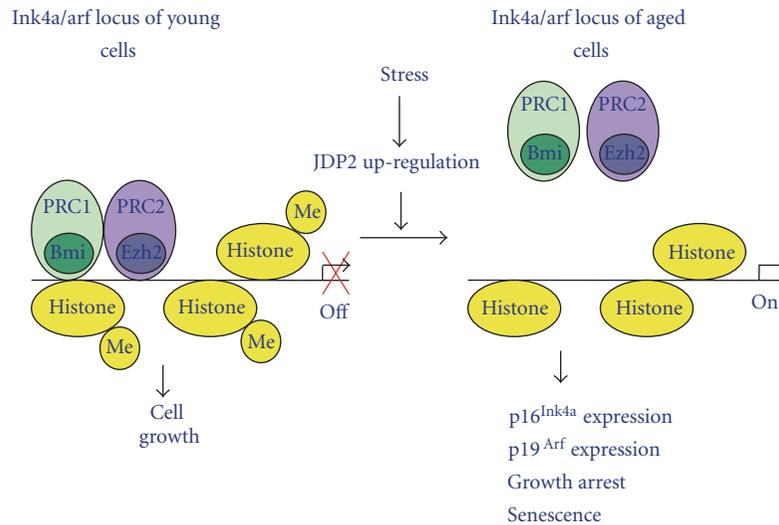


FIGURE 3: Proposed model of the epigenetic regulation of the expression of the genes for $p16^{Ink4a}$ and Arf by JDP2. The exposure of young MEF primary cells to aging stress leads to the accumulation of JDP2. JDP2 binds to histones and inhibits the methylation of H3K27 at the $p16^{Ink4a}/Arf$ locus. As a result, PRC-1 and PRC-2 fail to form stable repressive complexes and are released from the locus. The consequent expression of $p16^{Ink4a}$ and Arf in the aged cells leads to growth arrest and senescence stage.

Arf compared with those in 20% oxygen, whereas $Jdp2^{-/-}$ MEFs maintained low-level expression of $p16^{Ink4a}$ and Arf (Figure 3). These observations indicate that the aging-associated expression of $p16^{Ink4a}$ and Arf is dependent on oxygen stress and that JDP2 controls the expression of both $p16^{Ink4a}$ and Arf. We found no dramatic downregulation of the upstream repressors of $p16^{Ink4a}/Arf$, B lymphocyte molony murine leukemia virus insertion region 1 homology (Bmi1), and enhancer of zeta homolog 2 (Ezh2), in the absence of JDP2, suggesting that JDP2 does not regulate their expression. Interestingly, JDP2 expression in wild-type MEFs increased in the presence of 20% oxygen, but not in the presence of 3% oxygen, suggesting that its expression depends on oxygenic stress and that accumulated JDP2 may play a role

in the transcriptional activation of $p16^{Ink4a}/Arf$ (Figure 2). Studies based on ChIP have demonstrated that the methylation of H3K27 at the $p16^{Ink4a}/Arf$ locus was higher in $Jdp2^{-/-}$ MEFs than in wild-type MEFs and that the binding of polycomb repressor complex 1 (PRC1) and polycomb repressor complex 2 (PRC2) to the $p16^{Ink4a}$ and Arf promoters was more efficient in $Jdp2^{-/-}$ MEFs than in wild-type MEFs. These observations suggest that, in the absence of JDP2, H3K27 is methylated by PRC2, and the $p16^{Ink4a}/Arf$ locus is silenced by PRC1, whereas the increased expression of JDP2 helps to release PRC1 and PRC2 from the $p16^{Ink4a}/Arf$ locus, thereby reducing H3K27 methylation. Our data demonstrate that JDP2 is an important factor regulating cellular senescence. The loss of JDP2 allows MEFs to escape senescence,

and conversely, the overexpression of JDP2 induces cell-cycle arrest. The absence of JDP2 reduces the expression of both p16^{Ink4a} and Arf, which inhibit cell-cycle progression.

6.1. p16^{Ink4a} and the Rb Pathway. Genes that are essential for cell-cycle progression are transcribed at the beginning of G1 phase by transcription factors of the E2F family. E2F is controlled by Rb family of proteins, pRb, p107, and p130 [60, 61]. Early in G1, unphosphorylated Rb proteins bind to the E2F family of proteins and inactivate their function [62, 63]. During G1, the Rb proteins are inactivated by phosphorylation by Cdk4/6-cyclinD complexes, thereby allowing the transcription of E2F-dependent gene, including cyclin E. Upregulated cyclin E forms a complex with cdk2, that mediates the hyperphosphorylation of the Rb proteins, an essential requirement for the G1/S transition. p16^{Ink4a} is an allosteric inhibitor of cdk4/6. Binding to p16^{Ink4a} changes the conformation of cdk4/6, which prevents its interaction with cyclin D [64, 65]. Therefore, p16^{Ink4a} acts as an inhibitor of the cell cycle at G1 by modulating the Rb pathway. p16^{Ink4a} is often lost in a variety of human malignancies, including glioblastoma, melanoma, and pancreatic adenocarcinoma [66]. In contrast, the upregulation of p16^{Ink4a} induces the cell-cycle arrest and senescence [65, 66].

6.2. Arf and the p53 Pathway. p53 is known to mediate cell-cycle arrest, in G1 and G2, and apoptosis. A number of downstream targets of p53 are involved in these processes, including p21^{Cip/waf1} in G1 arrest [67], 14-3-3 sigma and growth arrest and DNA damage inducible gene 45 (GADD45) in G2 arrest [68, 69], and p21, Bax, PI-3 upregulated modulator of apoptosis (PUMA), Fas/Apo1, and Killer/DR5 in apoptosis [70–74]. p53 is regulated at the levels of protein stability and activity, and to some extent at transcription and translation [75, 76]. In unstressed cells, p53 protein levels are very low because its degradation is mediated by the E3 ubiquitin ligase activity of murine double minute 2 (MDM2), which targets p53 for ubiquitin-dependent proteolysis [77]. MDM2 is a transcriptional target of p53, so p53 directly activates the expression of its own negative regulator, producing a potent negative feedback regulatory loop [78]. There are several stress-responsive kinases, which, by phosphorylating p53, inhibit its degradation by MDM2 and increase its transcriptional activity [79–81]. DNA damage rapidly activates the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia-related (ATR) proteins, which phosphorylate the checkpoint kinases 1 and 2 (Chk1 and Chk2), which in turn propagate the signal to downstream effectors such as p53 [82, 83]. Both Chk1 and Chk2 phosphorylate p53 at Ser 20, which prevents the efficient recruitment of MDM2. Thus, p53 is stabilized and its expression level is increased in response to stress signaling. Arf is predominantly localized in the nucleoli and is stabilized by binding to nucleophosmin. In response to stress signaling, Arf is released from nucleophosmin and translocates to the nucleoplasm, where it interacts with MDM2, inhibits its E3 ubiquitin ligase activity, and blocks the nucleocytoplasmic shuttling of the MDM2-p53 complex.

Therefore, the consequences of the activation of Arf are the stabilization and activation of p53 [84, 85].

6.3. Senescence and Aging in Human and Mouse. Cellular senescence appears to be related to organismal aging. Cellular senescence involves processes that include telomere shortening, the accumulation of DNA damage, and the activation of the p16^{Ink4a}/Arf locus. The contributions of these factors to senescence seem to differ in humans and mice. Cultured mouse fibroblasts undergo senescence even when they have long telomeres and high telomerase activity. Senescence is abrogated by the loss of the p16^{Ink4a}/Arf locus [86]. In human cell cultures, the ectopic expression of telomerase is sufficient to overcome senescence by maintaining the length of the telomeres [87]. In mice, the maintenance of telomere length is important because telomerase deficiency shortens their lifespan and leads to premature aging [88–92]. The age-dependent accumulation of INK4A has been observed in the human kidney and skin [91], as well as in the majority of mouse tissues [92]. In oncogene-induced senescence, there is *in vivo* evidence that Arf is the important factor in the activation of p53 tumor suppression [91]. However, another study has shown that components of the DNA-damage-signaling cascade, including ATM and Chk2, are critical for the activation of p53 in response to oncogenic signals [93]. These differences between humans and mice could be attributable to species specificity and/or experimental conditions. Cellular senescence appears to be related to organismal aging because the same processes appear to be involved. Genetic variants of the p16^{Ink4a}/Arf locus are linked to age-associated disorders, such as general frailty, heart failure, and type 2 diabetes [94–99]. Mutations in telomerase or in proteins that affect telomerase activity are linked to premature human aging syndromes, including congenital dyskeratosis and aplastic anemia [100]. There are increases in DNA mutations, DNA oxidation, and chromosome loss during organismal aging. It seems reasonable to assume that all three factors, the activation of the p16^{Ink4a}/Arf locus, telomere shortening, and the accumulation of DNA damage, have cooperative effects on aging in physiological situations. Understanding the mechanisms of cellular senescence is currently of wide interest, and it is important that we identify new components of this process, such as JDP2.

7. Interaction of Transcription Factors with Histones and Nucleosomes

Similar to JDP2, other transcription factors or chromatin regulators are reported to have the binding affinities to histones and/or nucleosome. Transcription factor IIIA (TFIIIA) is a 40-kDa protein, with nine zinc finger domains, that binds specifically to the internal promoter of the gene for 5S RNA and to the N-terminal tail domains of histones H3 and H4, but not those of H2A and/or H2B, and directly modulates the ability of TFIIIA to bind nucleosomal DNA [101, 102].

A new class of HAT-regulatory proteins has been identified. These proteins block HAT activity via binding to and masking of the histone themselves. This class includes

the subunits of INHAT complex; TAF-1 α , TAF-1 β , and pp32 as well as ataxin 3; silencing mediator or retinoid receptor corepressor (SMART)/nuclear hormone receptor corepressor (NcoR); proline-glutamic acid- and leucine-rich protein 1 (PELP1) [103–107]. Thanatos-associated protein 7 (THAP7) is known to associate with TAF-1 β and to repress transcription by inhibition histone acetylation [108, 109]. A novel INHAT repressor (NIR) binds directly to nucleosomes and core histones and prevents acetylation by histone acetyltransferases, thus acting as a bona fide INHAT [110]. PU.1, a member of the Ets family of oncoproteins, inhibits CBP-mediated acetylation of globin transcription factor-1 (GATA-1) and erythroid Krüppel-like factor (EKLF), as well as of histones, and disrupts acetylation-dependent transcriptional events [111]. Moreover, PU.1 recruits the retinoblastoma tumor-suppressor protein, a histone methyltransferase (HMT) Suv39H, and heterochromatin protein 1 α (Hp1 α) [112]. Similarly, proliferating cell nuclear antigen binds to p300 to inhibit its HAT activity *in vitro* and to block HAT-dependent transcription *in vivo* [113]. TAF-1 β and pp32 have also been shown to interact with estrogen receptor (ER α), to inhibit the ER α -mediated activation of transcription, and to inhibit the activation of ER α acetylation by p300 [106, 107]. Moreover, TAF-1 β also interacts with the Sp-1 transcription factor and with KLF5, negatively regulating the binding to DNA and activation of transcription by these factors [114, 115]. Transcription factor NF- κ B p50 can accommodate distorted, bent DNA within the nucleosome [116], while DNA-binding domain (DBD) of c-Myb binds to the N-terminal tails of H3 and H3.3, and binding of c-Myb facilitates acetylation of histone tails [117]. Moreover, a kinetochore-null protein (KNL-2) with a c-Myb-like DNA-binding domain is specifically required for loading of centromere-specific variants of histone H3 (centromere protein A; CENP-A) in both nematodes and mammalian cells [118]. The recruitment of repressive macroH2A nucleosomes requires direct interactions between ATF-2 bound to a nearby AP-1 site and macroH2A, and recruitment is regulated by DNA-induced protein allostery [119]. Thus, the abovementioned sequence-specific DNA-binding factors might regulate transcription either via histone cores or tails, as well as via the structure of nucleosomes in conjunction with other proteins that bind to the chromatin. Indeed JDP2 is the first case to have both nucleosome assembly activity and DNA-binding activity [32] and inhibits the histone acetylation by p300/CBP [32, 33] as well as histone methylation [40] as described below.

8. Mechanism of JDP2-Mediated Regulation of C/EBP δ and p16^{Ink4a}/p19

Differentiation and senescence are associated with dynamic changes in gene expression, which are regulated by chromatin remodeling. Here, we have shown that the expression of JDP2 regulated the differentiation competent genes and the senescence competent genes such as C/EBP δ and p16^{Ink4a} and Arf in response to the induced hormone and accumulating oxidative stresses. In the case of suppression of

adipocyte differentiation by JDP2, how might JDP2 inhibit transcription of the C/EBP δ gene? It seems possible that, in response to signals that lead to differentiation, the C/EBP δ promoter might recruit transcription factors, including the coactivator p300, that mediate the acetylation of histones associated with the C/EBP δ gene to stimulate transcription of this gene. In the presence of JDP2, the acetylation of histone is inhibited and the expression of the C/EBP δ gene is suppressed during adipocyte differentiation, even in the presence of differentiation-inducing signals. Decreased expression of C/EBP δ results in less effective differentiation to adipocytes, a conclusion that is consistent with a previous report that deletion of the C/EBP δ gene results in the impairment of adipocyte differentiation [59]. By contrast, HDAC3 was not recruited by JDP2 to the promoter region of the C/EBP β gene, although the recruitment of HDAC3 to the C/EBP β gene was slightly enhanced during differentiation. In addition, the acetylation of histone H3 on the C/EBP β gene was less affected by differentiation-inducing stimuli. These observations suggest that histone acetylation might not play an important role in the transcriptional regulation of the C/EBP β gene and the HDAC3 might not be involved in such regulation. This hypothesis explains why the presence of JDP2 on the promoter had no effect on transcription. In conclusion, we propose that JDP2 acts as a negative molecular-switch in some, but not all, types of differentiation via the regulation of the expression of specific genes in concert with histone deacetylase HDAC3, as shown in the present study of adipocyte differentiation.

In the case of induction of cellular senescence by JDP2, we propose a model; the accumulation of oxidative stress and/or other environmental stimuli during aging upregulate JDP2 expression in primary untransformed cells. Increased JDP2 helps to remove PRC1 and PRC2, which are responsible for the methylation of histone H3, from the p16^{Ink4a}/Arf locus, leading to increased p16^{Ink4a} and Arf expression and entry into the senescence (Figure 4). There is some evidence that JDP2 acts as a tumor suppressor; JDP2 inhibits the Ras-dependent transformation of NIH3T3 cells [29], and JDP2 gene disruptions are often found in the lymphomas induced by insertional mutagenesis caused by the Moloney murine leukemia virus in MYC/Runx2 transgenic mice [27]. Here, we suggest that JDP2 not only inhibits the transformation of cells but also plays a role in the induction of cell senescence. Both functions of JDP2 might be important for its role in inhibiting tumor formation. Our findings also provide new insights into the molecular mechanisms, by which senescence is induced in the context of the epigenetic regulation of the p16^{Ink4a}/Arf locus. Recently, we reported that JDP2 regulates the expressions of cell-cycle regulators such as cyclin A2, cyclin E2, and p16^{Ink4a} and also affects the expression of p53 and p21 protein [120]. Thus, it is evident that JDP2 control the expression of cell-cycle regulators to induce the cell-cycle arrest via p53-p21 pathway and RB-p16 pathway.

9. Concluding Remarks

We characterized a sequence-specific DNA-binding protein as a nucleosome-assembly factor. Our finding should

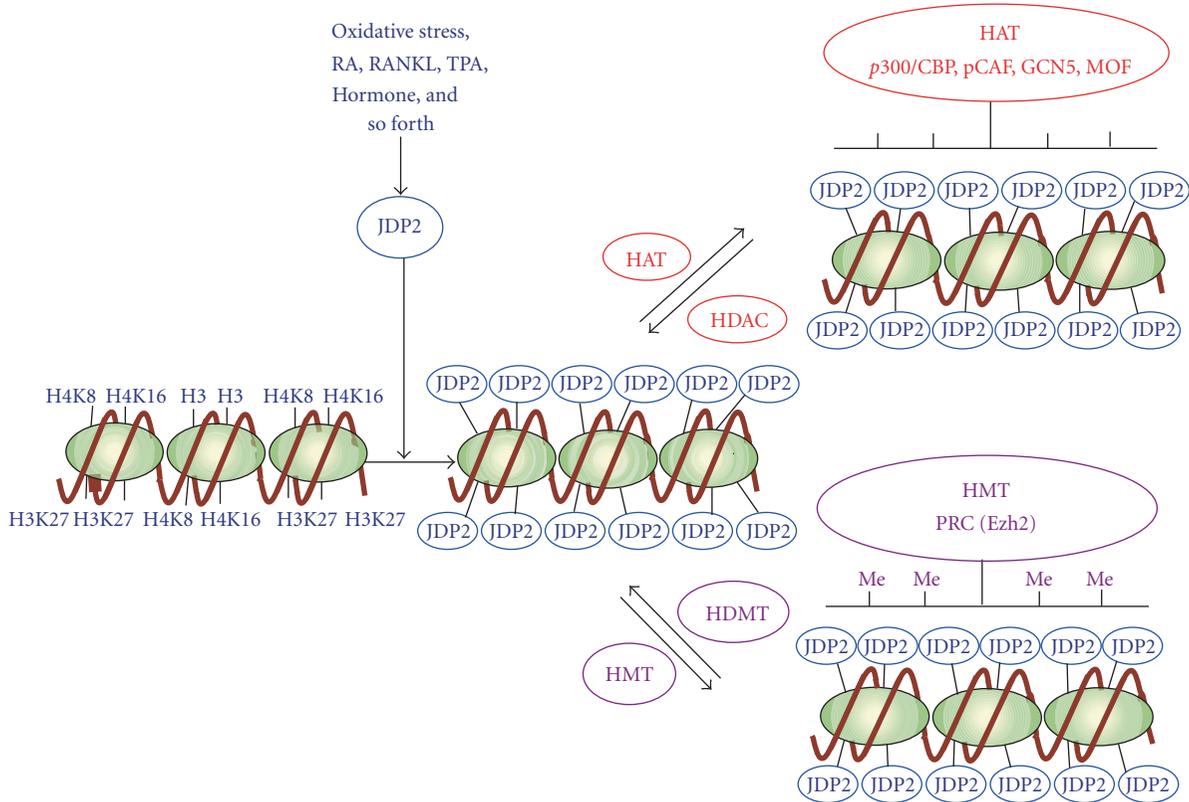


FIGURE 4: Model for the epigenetic regulation by JDP2. During the exposure of cells with oxidative stress, retinoic acid (RA), RANKL, TPA, and adipocyte inducing hormones, the histone H3, H4K8, and H4K16 as well as H3K27 were masked by JDP2 proteins and prevent the attack of histone modification enzymes like HAT (p300/CBP, pCAF, GCN5, MOF, etc.) and HMT (Ezh2 etc) in the cellular senescence and cell differentiation. This is a novel mechanism of JDP2 to inhibit the histone modification. The gene locus is either the $p16^{Ink4a}/Arf$ locus or C/EBP δ locus.

facilitate efforts to understand some aspects of nucleosome assembly and the remodeling of chromatin. Histone H3 seems to be recruited to target sites in a DNA synthesis-dependent manner via the interaction of chromatin assembly factor 1 (CAF-1) with proliferation cell nuclear antigen (PCNA); by contrast, a histone chaperone HIRA was reported to incorporate H3.3 in a DNA synthesis-independent manner [15]. The observation that histone H3.3 is found in several other subcomplex suggests that H3.3 might be recruited to different sites by different pathways. Identification of a gene-specific DNA-binding protein, namely JDP2, as a nucleosome-assembly factor suggests that H3.3 could be directly deposited at specific locations by site-specific DNA-binding proteins that also have histone-chaperone activity. It will be of interest to examine the binding preference of JDP2 for histone H3 as compared with H3.3.

Since JDP2 binds to core histone or nucleosome partially in DNA sequence-specific manner or histone subset-specific manner, the histone acetyltransferase or histone methyltransferase may not access to the nucleosome *in vitro* (Figure 4). However, we are not sure the case of *in vivo*. Chromatin immunoprecipitation assay (ChIP assay) demonstrated that JDP2 inhibited at least the acetylation of histone H4K8

and H4K16 although we cannot determine other precise residues of histone H3 acetylation (31). Moreover, JDP2 associated with histone H3K27 and blocked the methylation of histones (40). Thus, we assume that the interaction of JDP2 with nucleosome is DNA sequence or histone modification specific, and thus, only certain restricted set of histone might be associated with JDP2 *in vivo*. Addressing these precise functions in the context of epigenesis helps us to understand how senescence and differentiation, in a broader context, are regulated.

10. Future Prospects

Understanding how JDP2 promotes aging or cell differentiation, whether by inducing cellular senescence or decreasing the frequency of cell-cycle entry, is an important issue. The data demonstrating the increase in expression of $p16^{Ink4a}$ or C/EBP δ with aging (or oxygen stress) or hormone induction can be reconciled with two different models like “commitment model” and “threshold model.” With aging or hormone induction, stochastic activation of $p16^{Ink4a}$ or C/EBP δ expression could occur on a cell-by-cell basis in self-renewing compartments to induce commitment (commit-

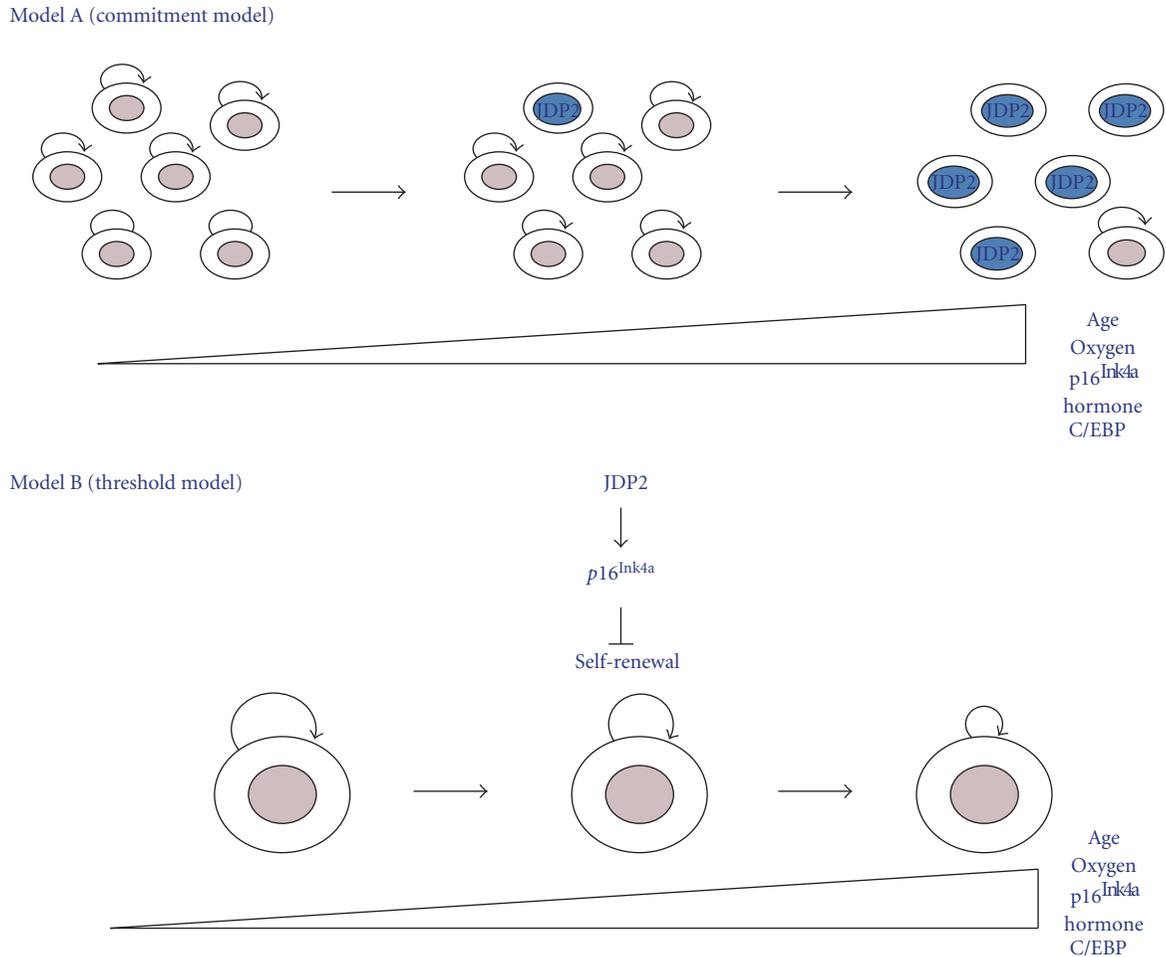


FIGURE 5: Model of role of JDP2 in cellular aging and cell differentiation. Self-renewing cells undergo repeated divisions, and p16^{Ink4a} or C/EBP δ expression increases with age, oxygen, and hormone, as a consequence of undefined stimuli. (a) Commitment model: p16^{Ink4a} or C/EBP δ expression occurs stochastically in a subpopulation of cells resulting in their senescence. In this model, the capacity for self-renewal of the noncommitted cells is not affected. (b) Threshold model: expression of p16^{Ink4a} or C/EBP δ increases or decreases, respectively, uniformly in the tissue specific. Self-renewing cells compartment is compromised over time. Self-renewal is indicated by curved arrows; committed cells are colored blue.

ment model), or expression could increase simultaneously with the majority of cells of a self-renewing compartment (threshold model) (Figure 5). In the latter case, self-renewal would be impaired by p16^{Ink4a} or C/EBP δ expression by decreased frequency of cell-cycle entry in the absence of senescence or cell differentiation.

Addressing this question has important implications for future “antiaging” therapies or “antidifferentiation” therapies. The model in which cell-cycle entry is decreased (threshold model) suggests that the age-induced defects in proliferation or the hormone-induced defects in proliferation could be improved merely by reducing p16^{Ink4a} levels or C/EBP δ levels or otherwise increasing cdk4/6 activity in these cells. The commitment model, however, suggests that the defects in self-renewal could only be remedied through more drastic self-renewing cells from an exogenous source. The understanding of the *in vivo* regulation of the Ink4a/Arf/Ink4b locus with aging (oxidative stress) or C/EBP δ locus with differentiation is needed further.

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Review Article

Regulating the Regulators: The Post-Translational Code of Class I HDAC1 and HDAC2

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Class I histone deacetylases (HDACs) are cellular enzymes expressed in many tissues and play crucial roles in differentiation, proliferation, and cancer. HDAC1 and HDAC2 in particular are highly homologous proteins that show redundant or specific roles in different cell types or in response to different stimuli and signaling pathways. The molecular details of this dual regulation are largely unknown. HDAC1 and HDAC2 are not only protein modifiers, but are in turn regulated by post-translational modifications (PTMs): phosphorylation, acetylation, ubiquitination, SUMOylation, nitrosylation, and carbonylation. Some of these PTMs occur and crosstalk specifically on HDAC1 or HDAC2, creating a rational “code” for a differential, context-related regulation. The global comprehension of this PTM code is central for dissecting the role of single HDAC1 and HDAC2 in physiology and pathology.

1. Histone Deacetylases: A Numerous Family with Many Biological Roles

Histone deacetylases (HDACs) are modification enzymes that catalyze the removal of acetyl molecules from ϵ -NH₃ groups of lysines, balancing the action of another family of enzymes, histone acetyl-transferases (HATs), which instead add acetyl groups.

They were first identified for deacetylating histones during chromatin remodeling, but many other nonhistone substrates have been characterized so far: transcription factors (p53 and Rb proteins), metabolic enzymes (Pyruvate kinase and Acetyl-CoA syntase), structural proteins (α -tubulin), enzymes involved in DNA dynamics (PCNA), as well as exogenous viral proteins (SV40 T antigen or HIV integrase) [1]. It is now clear that acetylation is a cellular broad-spectrum regulatory mechanism and HDACs are thought to be general deacetylation enzymes: in fact, it was recently proposed to rename them more properly “lysine deacetylases” (K[Lys]DAC) [2]. Nevertheless, since the name HDACs has historically entered into the acetylation field,

in this paper we will refer to them as histone deacetylases (HDACs).

Histone deacetylases constitute an ancient enzyme family, conserved in evolution from yeast to plants and animals [3]. HDAC-like proteins are found as well in Eubacteria and Archaeobacteria [4].

In humans, 18 HDACs have been described so far, divided into two families according to their mechanism of catalysis: zinc and NAD⁺ dependent.

The zinc-dependent HDACs belong to the classical Rpd3/Hda1 family, while the NAD⁺-dependent HDACs belong to the Sirtuin family.

Another complementary criterion of classification is based on sequence homology to the corresponding *Saccharomyces cerevisiae* proteins. According to this classification, HDACs are divided into four classes.

Class I HDACs (HDAC1, HDAC2, HDAC3, and HDAC8) are homologous to the yeast RPD3 protein. Class II includes HDACs homologous to the yeast HDA1 protein, and is further divided into two subclasses: class IIa (HDAC4, HDAC5, HDAC7, and HDAC9) and class IIb (HDAC6 and HDAC10) [5]. Class III corresponds to the NAD⁺-dependent Sirtuin

family and is composed of seven members, Sirt1-7 [6]. HDAC11 is the only member identified so far belonging to class IV; it is a zinc-dependent deacetylase sharing common features with both class I and class II HDACs [7].

The multiplicity of histone deacetylases reflects a diversification of functions in different tissues and biological processes. Sirtuins are mainly involved in metabolism and aging [6]; HDAC11 was shown to regulate the balance between immune activation and immune tolerance in CD4⁺ T-cell [8] and plays a role in oligodendrocyte differentiation [9].

Class II members have a variety of different roles in muscle, heart, and bone development and physiology, and they are expressed with a certain grade of tissue specificity [10]. Class II HDAC6 is the main cytosolic deacetylase controlling the cytoskeleton dynamics and chaperone functions through acetylation of α -tubulin and HSP90, respectively [11, 12].

On the contrary, class I HDACs are ubiquitously expressed in all tissue types [10]. HDAC8 plays an important role in smooth muscle cells, where it associates with α -actin and is essential for cell contractility [13]. Recently, a relation between HDAC8 transcriptional overexpression and advanced or metastasized stages of childhood neuroblastoma was described [14].

HDAC3 has a role in cell cycle progression and DNA damage response [15], as well as in spindle assembly checkpoint and sister chromatid cohesion [16]. Germline deletion of HDAC3 is lethal and embryos die before day 9.5, while conditional knockout of HDAC3 in MEF cells affects the transcription of a variety of genes involved in metabolism, cell cycle, apoptosis, development, and signal transduction [15]. Liver-specific knockout of HDAC3 leads to hypertrophy of hepatocytes and dysregulation of lipid metabolism [17].

HDAC1 and HDAC2, which are strictly related proteins, also have crucial roles in development and physiology, especially in the heart and nervous system [18]. They are also deeply involved in cellular proliferation, cell cycle, and apoptosis. HDAC1 has a pivotal role in the regulation of the cyclin-dependent kinase inhibitor p21 [19], and knockout mice for HDAC1 are embryonic lethal due to a strong impairment in cellular proliferation [20]. Depletion of HDAC1 results in perturbation of the cell cycle with loss of mitotic cells and increase in cell death in different human cancer cell lines [21]. In this paper, we will describe in detail the regulation of class I HDACs, and in particular of HDAC1 and HDAC2.

2. Regulation of Class I HDACs

The activity of class I HDACs in the cells is regulated through three main mechanisms: subcellular localization, association with other proteins into multisubunit complexes, and post-translational modifications (PTMs).

Class I HDACs are thought to be for the most part nuclear, with the exception of HDAC3, which possesses a nuclear export signal and can be found also in the cytoplasm [22, 23]. HDAC1, HDAC2, and HDAC3 are usually localized on chromatin, except during mitosis when all the three

HDACs are excluded from compact, mitotic chromosomes [15, 24]. The molecular details of how this exclusion is driven are still unknown.

HDAC1 and HDAC2, together with the histone binding proteins RbAP46 and RbAP48, are part of the catalytic core of many multiprotein transcriptional complexes, and this association stimulates their enzymatic activity [25]. The Sin3a and the NuRD complexes are broad-action modulators of gene transcription [26, 27] and bind a spectrum of different cofactors such as Sds3 [25] and SAP proteins [28], and Mi2, MTA2, and MBD3 [29], respectively. On the other hand, the REST/CoREST complex has more specific functions: it recruits HDAC1 and HDAC2 to suppress the transcription of neural genes in non-neural tissues [30, 31].

Recently, two other HDAC1/HDAC2 containing complexes were characterized; the SHIP and the NODE complexes. SHIP1 is a DNA-remodeling protein involved in chromatin dynamics during spermatogenesis: mass spectrometry analysis identified HDAC1 (but not HDAC2) as one of the components of the SHIP1 complexes [32].

NODE complex is peculiar of embryonic stem (ES) cells: HDAC1/HDAC2 containing complexes associate with the ES-specific transcription factors Nanog and Oct4, switching off specific target genes, and thus determining stem cell fate [33]. HDAC1/HDAC2 complexes are functionally distinct from HDAC3-containing complexes, such as the SMRT/N-CoR complex [34], thus suggesting a differentiation of biological roles between HDAC1/HDAC2 and HDAC3. To date, HDAC8 has not been reported to associate in macromolecular complexes.

HDAC1 and HDAC2 are not only protein-modifiers, but they are in their turn widely post-translationally modified, as it will be exhaustively described in the following paragraphs. Post-translational modifications (PTMs) represent a versatile and rapid mechanism to modulate protein functions and properties, such as enzymatic activity, subcellular localization, stability, and interaction with other binding partners in response to different extra- or intracellular stimuli. They can be either chemical moieties (acetylations, phosphorylations, methylations, nitrosylations, ADP-ribosylations, glycosylations, and carbonylations) [35] or proteins (ubiquitin, SUMO, NEDD8, FAT10, ISG15, and Atg8/Atg12) [36].

It is possible that the key to dissect single-HDAC-specific functions lies in decoding their PTM code, also, considering that, as it will be shown below, PTMs actively influence HDACs subcellular localization, enzymatic activity and complex formation. Given the above considerations, and given the strong association between HDAC1 and HDAC2 deregulation with the onset of many human diseases (from cancer to neuronal disorders, diabetes, and airway diseases), in this paper we will attempt to give a global overview of the post-translational code of HDAC1 and HDAC2.

3. HDAC1 and HDAC2: Equal but Not Too Much?

HDAC1 and HDAC2 are two highly related proteins, which are the result of the duplication of an ancient gene [5]. They

share 85% of global sequence identity, but this identity is not uniformly distributed; the dimerization and catalytic domains, which comprise the N-terminal two thirds of the proteins (amino acids 1-325), are 92% identical, while the C-terminal domains share 72% of identity. Even if the C-terminal domain has no catalytic activity *per se*, its deletion greatly reduces HDAC1 enzymatic activity [26], indicating that it plays an important role in the regulation of its functions. HDAC1 and HDAC2 are not only highly related in their structure and share many binding partners in macromolecular complexes (see above), but they have also redundant roles in a variety of biological processes. Conditional deletion of HDAC1 or HDAC2 was tolerated in different organs, such as the heart and the brain, if at least one allele of HDAC1 or HDAC2 was maintained [37], while concomitant deletion of both HDAC1 and HDAC2 caused mitotic catastrophe and cell death [38]. Differentiation of neuronal precursors into neurons requires the presence of either HDAC1 or HDAC2, strongly suggesting a compensatory effect [39]. Consistent with this observation, depletion of either HDAC1 or HDAC2 by RNA interference in cancer cell lines causes a compensatory upregulation of HDAC2 or HDAC1, respectively [21]. The redundancy of HDAC1 and HDAC2 has also been observed in the hematopoietic system. Levels and activities of HDAC1 and HDAC2 are critical for erythrocytic and megakaryocytic differentiation, and inactivation of HDAC1 and HDAC2 together results in severe thrombocytopenia due to apoptosis of megakaryocytes [40].

Nevertheless, the global picture is far more complex, because in other physiological settings HDAC1 and HDAC2 have specific and not compensatory functions. For example, knockout mice for HDAC1 die early in embryogenesis at E 9.5 [20], while knockout mice for HDAC2 survive until the perinatal period [37]. It was reported that in HeLa cervical cancer cells RNA interference for HDAC2 results in an increase in p21^{CIP/Waf1} expression [41], while in other cell types, like osteosarcoma U2OS cancer cells, p21^{CIP/Waf1} is upregulated also after HDAC1 depletion [21]. Finally, HDAC1, but not HDAC2, controls ES differentiation. Specific depletion of HDAC1 results in decrease activity of Sin3A, NuRD, and CoREST complexes, with concomitant decrease in acetylation of K56 of histone H3. Moreover, embryoid bodies derived from HDAC1-deprived (but not HDAC2-deprived) ES showed altered patterns of cardiomyocyte and neuronal markers [42].

The molecular rationale of when and why HDAC1 and HDAC2 have overlapping versus specific functions in different contexts is not yet clear. It is likely dependent on the cell or tissue types expressing specific combinations of proteins that can associate specifically with HDAC1 and HDAC2 or expressing different signaling pathways, in which PTMs likely act as principal effectors. It will be challenging to investigate whether a fine-tuned, differential PTM code might orchestrate differential functions of HDAC1 and HDAC2, thus creating a “single-HDAC regulatory code” despite their high overall similarity.

4. The Post-Translational Modification Code of HDAC1 and HDAC2

HDAC1 and HDAC2 are subjected to both chemical and protein PTMs. Although some PTMs occur in the catalytic domain, the majority of them occur in the less conserved C-terminal domain part (see Figures 1(a) and 1(b)). Known PTMs and their biological effects on HDAC1 and HDAC2 will be analyzed and compared.

4.1. Phosphorylation. Among the various post-translational modifications that occur on HDAC1 and HDAC2, phosphorylation is the best studied.

Phosphorylation in eukaryotes consists of the addition of a phosphate group (PO₄) on serine, threonine, or tyrosine residues by specific enzymes defined kinases. Phosphatases instead remove phosphate groups from proteins, balancing the action of the kinases [44].

HDAC1 is a substrate *in vitro* for phosphorylation by casein kinase II (CKII) and PKA but not by PKC, Cdc2, or MAP kinases [45]. HDAC2 is also phosphorylated by CKII *in vitro*, but unlike HDAC1, it is not a substrate for PKA [46].

The main kinase responsible *in vivo* for HDAC1 and HDAC2 phosphorylation is casein kinase II (CKII), which is also found in the HDAC1/HDAC2 containing complexes such as Sin3 and NuRD complexes [47]. It phosphorylates HDAC1 on serines 421 and 423, and mutations in these sites determine reduction of deacetylating and transcriptional repression activities and interaction with RbAP48, Sin3a, CoREST, and MTA-2 [48].

It was also reported that CKII-dependent phosphorylation on HDAC1 is constitutive throughout the cell cycle, but dispensable for its intrinsic activity *in vitro* [49]. This was also confirmed in another study where HDAC1 treated with calf intestinal phosphatase is still able to deacetylate a histone H4-derived peptide [45]. An additional phosphorylation site on tyrosine 221 HDAC1 was discovered through mass spectrometry screenings [46]: it is the only example of tyrosine phosphorylation on HDAC1, but without any evident role yet.

CKII also widely phosphorylates HDAC2 *in vitro* and *in vivo* on serines 422 and 424, homologues of HDAC1 CKII-sites, and on serine 394, which seems to be the main phosphorylation site for HDAC2 [50]. Interestingly, one phosphorylation site was found in the region 386–409 of HDAC1 [45] and recent high throughput mass spectrometry analysis has identified the correspondent serine 393 of HDAC1 as a new phosphorylation site, but the role is still to be defined [51].

It was also reported for HDAC2 a fourth phosphorylation site on serine 411, but it seems to be independent from CKII action and its role is still unknown. In analogy with HDAC1, HDAC2 catalytic activity as well as the binding with Sin3a and Mi2 is promoted by CKII-dependent phosphorylation, while it does not affect the binding of HDAC2 with HDAC1 [50]. This is consistent with a previous study, which identified the interaction domain of HDAC1 with HDAC2 in the N-terminal region of HDAC1 [43].

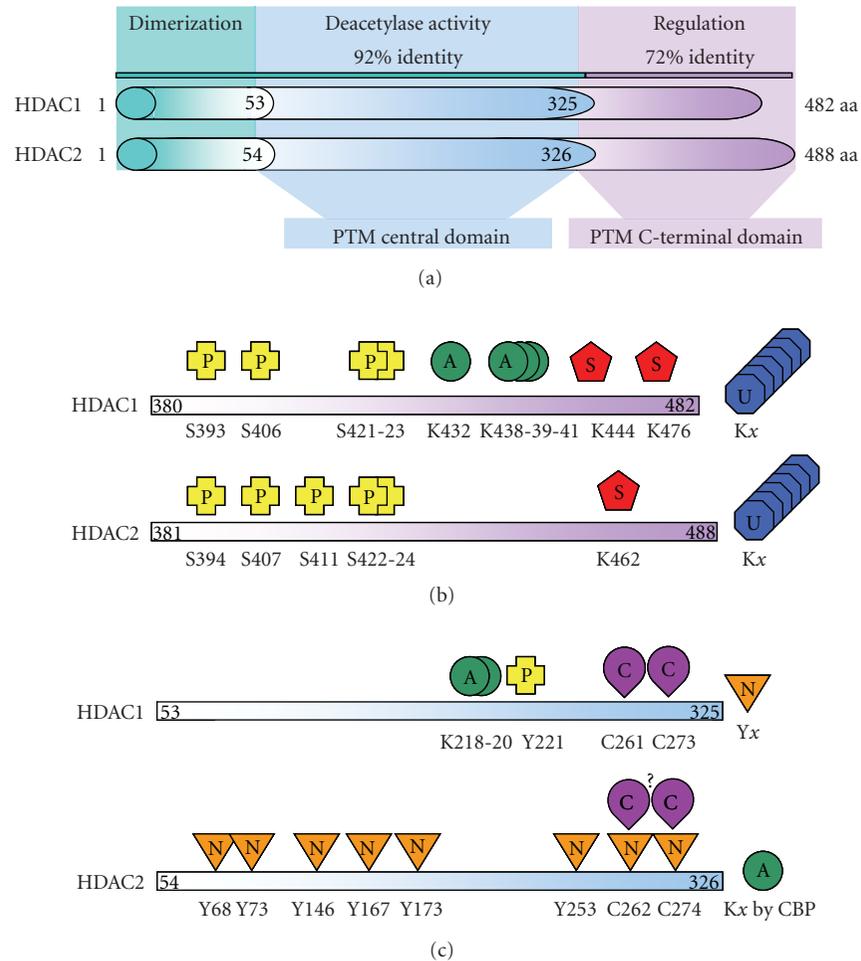


FIGURE 1: Schematic representation of the PTMs spatial distribution of HDAC1 and HDAC2. (a) Organization of the functional domains of HDAC1 and HDAC2, as described in [43]. Numbers indicate the corresponding amino acidic (aa) positions. Percentages of identity were calculated by BLAST alignment of HDAC1 (CAG46518.1) and HDAC2 (AAH31055.2) protein sequences. (b) and (c) Visual comparison of the different PTMs occurring on HDAC1 and HDAC2 in the C-terminal (B) and central (C) domains. Different PTMs are illustrated by different colors and letters: P (yellow): phosphorylation, A (green): acetylation, U (blue): ubiquitination, S (red): SUMOylation, N (orange): nitrosylation, and C (lilac): carbonylation. The amino acidic sites of modification are indicated by the number of their position in the protein sequence: S: serine, K: lysine, Y: tyrosine, C: cysteine, and CBP: CBP histone acetyltransferase. When the precise position of a modified residue is not known, the amino acidic site is followed by an *x*. The question mark (?) is used when the precise amino acidic site has not been formally identified. The amino acids at the boundaries of the two domains are reported.

CKII-driven phosphorylation of HDAC2 has been reported to regulate its differential distribution on specific functional areas of chromatin. In particular, unmodified HDAC2 is associated with coding region of genes contributing to active transcription, while the phosphorylated population is more involved in gene regulation, being preferentially recruited to gene promoters by RbAP48 into Sin3 and NuRD complexes [47, 52].

Phosphorylation of HDAC2 on serine 424 is also involved in the response of vascular smooth muscle cells to stimulation with all-trans retinoic acid (ATRA). Upon stimulation, a signaling cascade operating via the JNK kinase determines an increase in HDAC2 phosphorylation, which in turn causes the dissociation of HDAC2 from its binding partner KLF4. The consequent increase in KLF4 acetylation leads to the

expression of differentiation marker genes such as SM22 α [53].

CKII-dependent phosphorylation of HDAC2 on serines 394, 411, 422, and 424 increases upon exposure to cigarette smoke (CS) and is related to an increase in ubiquitination and degradation by the proteasome pathway in macrophage, human bronchial epithelial and small airways epithelial cells as well as in the lung of living mice [54]. Interestingly, single mutations of serine 394 or serine 424 did not affect global HDAC2 phosphorylation after CS exposure as the quadruple mutant did, suggesting a certain grade of redundancy between the sites [55]. Since HDAC2 suppresses expression of genes involved in inflammation, and cigarette smoke causes oxidative stress and is the primary risk factor for chronic obstructive pulmonary disease (COPD), the

elucidation of this mechanism may have clinical relevance in the steroid resistance observed in COPD patients and in smokers affected by severe asthma.

The biological relevance of HDAC1 and HDAC2 phosphorylation is also depicted by the fact that some viruses induce their hyperphosphorylation in the early phase of infection. U_s3 kinase from human Herpes Simplex Virus 1 [56] and its homologue ORF66 kinase from Varicella Zoster Virus [57] both induce, even if indirectly, hyperphosphorylation of HDAC1 and HDAC2. The target sites of ORF66-induced phosphorylation were identified as serine 406 of HDAC1 and serine 407 of HDAC2 [57]. Recently, also the nonhuman herpesvirus Pseudorabies Virus (PRV) U_s3 kinase was shown to induce phosphorylation of HDAC2 on serine 407, indicating a conserved effect of many herpesviruses on this deacetylase [58]. The kinase(s) directly responsible for HDAC1 and HDAC2 phosphorylations *in vivo* is unknown; CKII seems unlikely to be the cellular kinase involved, since it requires the acidic consensus sequence S/T-X-X-E [59], not present in the region encompassing serines 406 of HDAC1 and 407 of HDAC2.

CKII-dependent phosphorylation of HDAC1 and HDAC2 is also increased in response to hypoxic conditions, and this correlates with an increase in HDAC enzymatic activity [60]. These studies suggest that phosphorylation is a key signaling factor of different pathways that converge on HDAC1 and HDAC2 to carry on biological responses.

Finally, it was shown that proper histone deacetylation is required for meiotic resumption in porcine oocytes, and phosphorylation of HDAC1 was observed to rapidly change during oocyte maturation. The role of HDAC1 phosphorylation in the meiotic process has not been investigated in details but it is another indication of the variety of biological processes where phosphorylation of HDAC1 is involved [61].

Protein phosphatases are as important as protein kinases in regulating the phosphorylation status of substrates. HDAC1 and HDAC2 are dephosphorylated *in vitro* by protein phosphatase 1 (PP1) and λ -phosphatase [62]. Treatment of different cell lines with the phosphatase inhibitor okadaic acid induces the appearance of hyperphosphorylated forms of HDAC1 and HDAC2, associated with a slightly decrease in HDAC activity and loss of binding between HDAC1 and Sin3a and HDAC1/HDAC2 with the transcriptional partner YY1 [62]. Apparently, this seems in conflict with the work described above showing that phosphorylation positively regulates HDAC1 and HDAC2 enzymatic activities and binding properties. It is worth pointing out that the different studies used different approaches (site-directed mutagenesis versus treatment with a general phosphatase inhibitor), and, considering the complexity of the system of modifications acting on HDAC1 and HDAC2, this could at least in part account for the apparent discrepancy. HDAC1 and HDAC2 interact with protein phosphatases *in vivo* [63, 64]; treatment of cells with the pan HDAC inhibitor trichostatin A (TSA) [65] causes destruction of a HDAC1-PP1 complex [66, 67], but the catalytically dead mutant of HDAC1 (HDAC1 H141A) still retained binding with PP1 [63]. This example shows that the use of aspecific, general inhibitors of the enzymatic activity of an enzyme (TSA or okadaic acid) may

have different effects compared to the use of more specific point mutants.

HDAC1, and to a lesser extent HDAC2, were found in complex with PP1 and the transcription factor CREB. HDAC1 and PP1 cooperate to deacetylate histones and to dephosphorylate CREB, thereby silencing CREB-driven transcription in c-AMP unstimulated cells or in later times after stimulation, contributing to signal attenuation [63]. HDAC1 and PP1 are also part of a complex with the Rb protein, which is dissociated in response to ionizing radiations in an ATM-dependent pathway [68]. In brain cells, PP1-HDAC1 containing complexes are central actors in the modulation of the epigenetic histone code at the promoters of genes implied in memory formation [69].

Although it is likely that many other phosphorylation sites will be identified in the future, phosphorylation of HDAC1 and HDAC2 has been biochemically well characterized so far, but a whole comprehension of its biological roles is still elusive. It is likely that the scenario will become more and more sophisticated in the future, as long as new PTMs and the crosstalk between them will be discovered.

4.2. Acetylation. Histone deacetylases are the main actors in the acetylation network that takes place in cells, but they are themselves regulated by acetylation.

HDAC1 can be acetylated on six lysines: K218, K220, K432, K438, K439, and K441. It was shown that in cells, a subpopulation of HDAC1 is acetylated by the acetyltransferase p300 at the promoter of glucocorticoid-receptor (GR) driven genes one hour after binding of the hormone. Acetylation of HDAC1 reduces dramatically its enzymatic and repression activities *in vitro* and *in vivo*. HDAC1 acts as a coactivator of GR activation of promoters; consequently, a mechanism was proposed that after gene activation induced by the hormone, p300 is recruited at the promoter, and HDAC1 becomes acetylated and inactivated to allow the attenuation of the signal and the restoration of the transcriptional steady-state levels [70].

On the contrary, HDAC2 is not acetylated *in vitro* by p300 even if five out of the six lysines acetylated on HDAC1 are conserved [71]. The only lysine not conserved is K432 (arginine R433 in HDAC2), which was shown to be the critical residue for HDAC1 acetylation [70]. The conversion of R433 of HDAC2 into lysine rendered HDAC2 acetylated by p300 *in vitro* although to a lesser extent compared to HDAC1 [71]. Strikingly, the substitution of the C-terminal tail of HDAC2 with the tail of HDAC1 determined a full acetylation of HDAC2, compared to HDAC1 wild type. Vice versa, HDAC1 bearing the C-terminal tail of HDAC2 was totally refractory to acetylation by p300. Acetylation of HDAC1 not only reduces the activity of HDAC1 itself, but acts in trans also on HDAC2 both *in vitro* and *in vivo* on the HDAC1/HDAC2 heterodimers of the Sin3a, NuRD, and CoREST complexes. These observations clearly show how single differences in key residues of HDAC1 and HDAC2 can affect their PTM code. More importantly, they highlight the crucial role of the C-terminal region in the differential regulations of the enzymatic activity of the two enzymes.

Based on these experimental data, we can speculate that C-terminal domain of HDAC1 and HDAC2, which is not conserved in the other, more divergent class I HDAC3 and HDAC8 [3, 72], has evolved as a molecular platform to specify and finely tune the functions of two highly related proteins in the complex networks of mammalian systems.

Recent work highlights that HDAC2 binds the acetyltransferase CBP only in response to cigarette smoke treatment and is, therefore, acetylated. This acetylation relies on previous CKII-dependent phosphorylation, since the phosphorylation quadruple mutant is less acetylated after CS treatment. This is one of the first instances of a crosstalk between different PTMs acting on HDAC2 (see Figure 2). Opposite to what observed for acetylation of HDAC1 by p300, acetylation of HDAC2 by CBP increases its transrepressional activity in luciferase-reporter assays [55]. These observations also suggest that the action of different HATs on single HDACs may result in different biological outcomes, creating a multilevel network of regulation.

4.3. Ubiquitination. Ubiquitin (Ub) is a small protein conjugated to lysine residues of target substrates through an isopeptide bond, as a single monomer or as a polyubiquitin chain. The reaction involves a well-defined three-step enzymatic cascade (E1 activating enzymes, E2 conjugating enzymes and E3 ubiquitin ligases). Polyubiquitination mainly targets proteins for degradation via the proteasome machinery, while monoubiquitination acts as a signal for different biological outputs [36].

Both HDAC1 and HDAC2 have been reported to be polyubiquitinated *in vitro* and *in vivo* [75–77]. HDAC1 is ubiquitinated and rapidly degraded by a proteasome-dependent mechanism in presence of the antiproliferative agent quinidine [78]. Gaughan and colleagues were the first to describe a Ub E3 ligase for HDAC1 *in vivo* [79]. They showed that HDAC1 is found in complex with the androgen receptor (AR) protein and the Ub E3 ligase Mdm2 at the promoter of AR-driven genes upon hormone treatment in prostate cancer cells. Ubiquitinated forms of HDAC1 were detected only after hormone treatment and were correlated to the Ub E3 ligase activity of Mdm2. Mdm2 also ubiquitinates AR protein, and HDAC1 cooperates with Mdm2 possibly through deacetylation of critical lysines of AR protein and thus reducing transcription of the downstream gene. HDAC1 ubiquitination by Mdm2 may represent a further level of fine regulation at the promoter, reducing HDAC1 protein levels and likely exposing the promoter for a second round of active transcription.

HDAC1 ubiquitination and proteasome-dependent degradation was recently shown to have significant biological relevance. Work by Oh and colleagues identified a functional interaction between HDAC1 and the Chfr Ub ligase. Chfr downregulates HDAC1 levels by directly binding and ubiquitinating HDAC1. This mechanism correlates with increased invasiveness and metastatic potential of prostate and breast cancer cell lines [80]. Selective ubiquitination and depletion of HDAC1, but not of other class I HDACs including HDAC2, is also a

critical step of the proinflammation response activated by tumor necrosis factor- α (TNF α) through the IKK2 signaling pathway [81]. Upon binding of TNF α to its receptor, the IKK complex (IKK1/IKK2/NEMO) phosphorylates the I κ B α protein, which then releases NF- κ B protein. Free NF- κ B translocates from the cytoplasm to the nucleus where it acts as a transcription factor activating a plethora of inflammatory, antiapoptotic and proliferative genes. HDAC1 is ubiquitinated after TNF α treatment and degraded via the proteasome machinery, only in the presence of an active IKK2. As a result, a complete loss of HDAC1 occupancy at the promoter of p21^{WAF/CIP1} gene is observed. These results provide evidence of a link between inflammatory signaling pathways and modulation of chromatin transcription through PTMs on histone deacetylases.

HDAC2 is specifically ubiquitinated for proteasomal degradation by the Ubc8 E2 conjugase and the RLIM E3 ligase upon treatment with valproic acid, an inhibitor of class I and IIa HDAC enzymatic activities [76].

Interestingly, valproic acid does not affect HDAC1 stability in endometrial stromal sarcoma cells [82]. This work, together with the study by Vashisht Gopal and collaborators [81], suggests the existence of signaling pathways able to discriminate between HDAC1 and HDAC2 at a PTM level to achieve different specific responses in different cellular contexts.

Finally, HDAC2 was shown to be phosphorylated and degraded by the proteasome in bronchial cells upon treatment with cigarette smoke. A possible correlation with the CKII-dependent phosphorylation was proposed [54] (see Figure 2).

4.4. SUMOylation. The conjugation of SUMO proteins (SUMO1, SUMO2, and SUMO3) to substrates mirrors ubiquitin conjugation; an E1-E2-E3 enzymatic cascade adds SUMO proteins to lysines of target substrates. Differently from ubiquitination, the SUMOylation consensus site Φ -K-X-E (where Φ is an aliphatic residue, preferably L, I or V, K is lysine, X is any residue and E is glutamate) is frequently, even though not always, found on substrates [83].

HDAC1 is SUMOylated by SUMO1 *in vivo* and *in vitro*, and by SUMO2/3 *in vitro* [75, 84, 85]. Two independent works mapped the SUMOylation sites of HDAC1 on lysines K444 and K476, but its biological role is still under investigation. We did not observe an effect of the double SUMOylation mutant K444R, K476R (2R) on HDAC1 enzymatic activity and transcriptional repression [84], while work from De Pinho's laboratory reported a 60% reduction of transcriptional repression of the 2R HDAC1 mutant, even if the binding with the Sin3a protein was unaffected.

Moreover, overexpression of wild type HDAC1, but not of the 2R mutant, induced accumulation of cells in the G2 phase of the cell cycle, suggesting that the SUMOylation mutant of HDAC1 is impaired in some biological functions [75]. Furthermore, other studies have reported that SUMO modification promotes transcriptional repression of HDAC1/HDAC2 containing complexes, such as Elk1 or Mi-2/NuRD [86, 87].

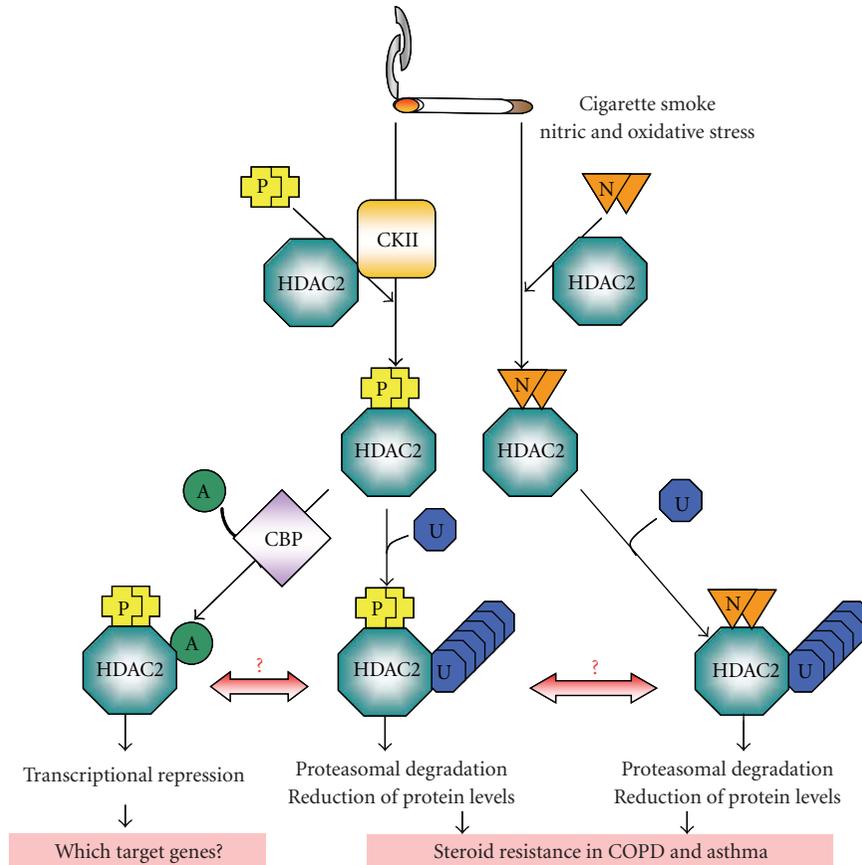


FIGURE 2: Schematic illustration of the crosstalk between different PTMs on HDAC2 in response to oxidative or nitric stress stimuli. Oxidative or nitric stimuli evoke a complex response in terms of PTMs on HDAC2 that results in a specific biological output [54, 55, 73, 74]. Different PTMs are illustrated by different colors and letters: P (yellow): phosphorylation, A (green): acetylation, U (blue): ubiquitination, and N (orange): nitrosylation. The modification enzymes, when known, are also reported: CKII: casein kinase II, and CBP: CBP histone acetyltransferase. When a mechanistic relation between two different PTMs events is not known, the question mark (?) has been used.

The ability of the 2R HDAC1 mutant to be polyubiquitinated was also evaluated: although both the wild type and the 2R mutant show consistent ubiquitination, the latter appeared to be ubiquitinated to a lesser extent [75]. If this is due to a mutation of the two lysines, which could serve as target residues for conjugation of ubiquitin, or to a more complex cross regulation between SUMOylation and ubiquitination still remains unknown. It is also worth noticing that SUMOylation of the phospho-null mutant S421A, S423A of HDAC1 was increased respect to the wild-type protein [84]. This finding has not been further investigated, but it may suggest a negative relationship between CKII phosphorylation and SUMOylation.

SUMO proteins are reversibly detached from substrates through the action of SUMO proteases called SENPs (SEN1–7 in mammals) [88].

HDAC1 can be deSUMOylated *in vivo* by SENP1 [89], a SUMO protease able to cleave both conjugated SUMO1 and SUMO2, but not SUMO3 [90].

HDAC1 represses AR-gene transcription [91] and SENP1 was shown to enhance ligand-dependent transcription of androgen-receptor (AR) driven genes. HDAC1 and HDAC2

were found to complex with SENP1 at the promoter of AR-genes and the transcription-promoting action of SENP1 is carried out through the deSUMOylation of HDAC1. Consistent with this model and with previous results [75], wild type HDAC1 can repress AR-activity up to 90% while the 2R HDAC1 mutant only to 60% [89].

Up to date, SUMOylation of HDAC2 has not been reported in the literature, but unpublished data in our laboratory have identified lysine 462 as a target site for SUMOylation of HDAC2 by SUMO1 both *in vitro* and *in vivo* (C.V. Segre' and S. Chiocca, *unpublished data*). It is worth noticing that this SUMO consensus site for HDAC2 is not conserved exactly as in HDAC1, but its position in the protein sequence is intermediate between the positions of the two SUMO sites on HDAC1. This might suggest slight differences in the regulation of HDAC1 and HDAC2 by SUMOylation.

Proteins can interact with SUMO also in a noncovalent manner, through a SUMO-Interacting-Motif (SIM) [92]. HDAC2 in particular is recruited by different SUMO-bound transcriptional factors, such as Elk1 and CBP to repress transcription of target genes [86, 93]. SUMOylation of Elk1

in vitro specifically promotes HDAC2 binding, suggesting the presence of a SIM motif on HDAC2. Finally, recent evidence indicates that HDAC2 promotes SUMOylation of the translation initiation factor eIF4E, modulating the translation of a specific subset of mRNAs [94].

4.5. Nitrosylation. Nitrosylation is a protein modification in which a nitrosyl group (NO) is post-translationally added to an amino acid residue. Nitric oxide (NO) production in cells is catalyzed by enzymes called nitric oxide synthases (NOS), and it represents a key second messenger in a variety of biological processes including neurotransmission, immune defense, apoptosis, and cell motility [95]. NO can rapidly react with the superoxide anion O_2^- producing the highly oxidant specie peroxynitrite ($ONOO^-$), which in turn reacts with amino acids and generates a nitrosylated residue.

Tyrosine nitrosylation was observed in HDAC1, HDAC2, and also HDAC3 in macrophage cells after exposure to cigarette smoke [96]. Another study using human alveolar epithelial cancer cells showed that different tyrosine residues of HDAC2 are nitrosylated after oxidative and nitric stress: Y68, Y73, Y167, Y146, Y173, and Y253. Interestingly, mutation of Y253 abolished the proteasomal-dependent degradation induced by the peroxynitrite generator SIN-1 [73], thus suggesting that tyrosine nitrosylation of HDAC2 is a stress signal to drive specifically its degradation.

In neurons, HDAC2 is s-nitrosylated on cysteines 262 and 274 in response to BDNF-NO signaling. Null mutations of these cysteines did not dramatically affect HDAC2 enzymatic activity, but s-nitrosylation caused dissociation of HDAC2 from the promoters of some neuronal specific genes, such as Fos, Egr1, Vgf, and Nos1 with concomitant increase of histone acetylation and transcriptional activation [97]. S-nitrosylation of HDAC2 also plays a role in the pathogenesis of Duchenne muscular dystrophy, where it is correlated with a decrease in HDAC2 enzymatic activity and partial rescue of the myotube differentiation abilities of cells, which are lost in the pathology [98].

Recent work showed that s-nitrosylation of HDAC2 in macrophages following lipopolysaccharide (LPS) stimulation affects the stability of the binding between HDAC2 and MTA1, with consequent dissociation of the NuRD complex from chromatin and activation of transcription of some inflammatory genes like IL1 β , TNF α and MIP2 [99].

This is another indication that PTMs of HDAC1 and HDAC2 are the first and most important level of regulation, influencing also other levels of regulation such as multiprotein complex formation.

Interestingly, no s-nitrosylation has been reported for HDAC1 or HDAC3 [98], even if the two cysteines are well conserved in all three proteins [48].

These studies suggest an emerging role of s-nitrosylation in regulating the chromatin-associated dynamics of HDAC2, in a very specific way also compared to other class I HDACs, such as the highly related HDAC1.

4.6. Carbonylation. Carbonylation (or alkylation) is a distinctive PTM of redox signaling pathways occurring in cells

after oxidative stress. Reactive carbonyl species (RCS) derive from peroxydation of lipids (especially arachidonic acid), which generate α - β -unsaturated aldehydes (e.g., 4-hydroxy-2-nonenal) and α - β -unsaturated ketones (e.g., cyclopentenone prostaglandis). Covalent binding of RCS on cysteinyl thiols of substrate proteins is termed carbonylation [74].

HDAC1, HDAC2, and HDAC3, but not the other class I HDAC8, are carbonylated after treatment with cyclopentenone prostaglandis [100]. The cysteines involved in this PTM have been identified for HDAC1 as Cys 261 and Cys 273, which are conserved in both HDAC2 and HDAC3, but not in HDAC8, which in fact is not modified. Carbonylation of HDAC1 does not impair its intrinsic enzymatic activity, but disrupts its interactions with binding partners and histone substrates *in vivo*. As a consequence, increased acetylation of lysine 9 of histone H3 was observed with concomitant induction of transcription of some HDAC1-repressed genes, such as HO-1, Gadd45, and HSP70. This paper represents one of the first reports linking redox signaling species such as RCS to modification in chromatin states through a direct modulation of histone deacetylases, resulting in the expression of genes involved in the response of cells to oxidative stresses.

The authors did not formally demonstrate that the corresponding cysteines of HDAC2 and HDAC3 are the carbonylation sites as for HDAC1, but speculated that they are likely to be the target residues of the cyclopentenone prostaglandis covalent binding, based on the evolutionary conservation with HDAC1. It is a reasonable speculation considering that for other PTMs, such as phosphorylation, a similar pattern of modification occurs on corresponding residues of HDAC1 and HDAC2 (see Table 1 and Figure 1). Nevertheless, in the case of redox signaling-associated PTMs, the picture might be more complicated. In fact, HDAC2 is modified by NO on the conserved Cys 262 and Cys 274 [97], but HDAC1 and HDAC3 are not [98], indicating that the simple conservation of the same amino acidic stretch is not always sufficient to have the same PTM. It is likely that other factors, such as cell-type specific interactors, contribute to the final PTM pattern of single HDACs.

5. Reassembling the Pieces of the Puzzle: The Integration of the Code

The bulk of work on HDAC1 and HDAC2 PTMs (summarized in Table 1) seems to unveil a complex “code” by which the two proteins can be differentially regulated, despite their high overall similarity and partially redundant roles.

Two “modular” areas of PTMs can be distinguished on HDAC1 and HDAC2: a “tyrosine-cysteine modification” central domain and a “serine-lysine modification” C-terminal domain (see Figures 1(a) and 1(b)).

The vast majority of the modifications reported in the central domain of the two HDACs are nitrosylations and carbonylations in consequence of inflammatory/oxidative stresses; it could be speculated that the central domains of the deacetylases act as a platform for the integration of signals in response to stress stimuli (such as cigarette smoke, LPS, or

TABLE 1: Summary of the PTM code on HDAC1 and HDAC2. The modification/demodification enzymes, amino acidic sites, and the effects and/or the biological relevance caused by the modification and the citations (refs) are reported.

		Phosphorylation		
	Kinase/ Phosphatase	Sites	Effects/biological relevance	Refs
HDAC1	CKII	S421, S423 <i>in vivo</i> and <i>in vitro</i>	Stimulation of catalytic activity and binding properties	[45, 48, 49]
	PKA	<i>in vitro</i>	n.r.	[45]
	PKC, Cdc2 and MAP kinase	NOT phosphorylated <i>in vitro</i>	/	[45]
	n.r.	S406 <i>in vivo</i>	Consequent to viral infection	[56, 57]
	n.r.	S393	identified by mass spectrometry analysis	[45, 46, 51]
	n.r.	Y221	identified by mass spectrometry analysis	[46]
	Protein phosphatase 1 λ -phosphatase	<i>in vitro</i> <i>in vitro</i>	n.r. n.r.	[62] [62]
HDAC2	CKII	S394, S422, S424 <i>in vivo</i> and <i>in vitro</i>	Stimulation of catalytic activity and binding properties	[50]
			Differential localization on chromatin	[47, 52]
			Transcriptional regulation of SM22 α in vascular cells	[53]
			Transcriptional regulation in bronchial cells	[54, 55]
	PKA, PKC, and PKG	NOT phosphorylated <i>in vitro</i>	/	[50]
	n.r.	S411 <i>in vivo</i>	n.r.	[50]
	n.r.	S407 <i>in vivo</i>	Consequent to viral infection	[56–58]
Protein phosphatase 1 λ -phosphatase	<i>in vitro</i> <i>in vitro</i>	n.r. n.r.	[62] [62]	
Acetylation				
	HAT/HDAC	Sites	Effects/biological relevance	Refs
HDAC1	p300 (HAT)	K218, K220, K432, K438, K439, K441	Transcriptional attenuation of GR-driven genes	[70]
	p300 (HAT)	<i>in vivo</i> and <i>in vitro</i>	Reduction of catalytic activity and binding properties	[71]
HDAC2	CBP (HAT)	n.r.	Increase of transcriptional repressive activity	[55]
	p300 (HAT)	NOT acetylated	/	[71]
Ubiquitination				
	E1/E2/E3	Sites	Effects/biological relevance	Refs
HDAC1	Mdm2 E3 ligase	n.r.	Modulation of transcription at AR-driven promoters	[79]
	Chfr E3 ligase	n.r.	Increase of invasiveness and metastatic potential of cancer cells	[80]
	n.r.	n.r.	Response to the antiproliferative agent quinidine	[78]
	n.r.	n.r.	Response to TNF α stimulation via IKK2 signalling	[81]
HDAC2	Ubc8 E2 conjugase	n.r.	Response to valproic acid treatment	[76]
	RLIM E3 ligase	n.r.		
	n.r.	n.r.	Response to oxidative and nitric stress	[54, 73]
SUMOylation				
	SUMO protein/proteases	Sites	Effects/biological relevance	Refs
HDAC1	SUMO1	K444, K476 <i>in vivo</i> and <i>in vitro</i>	Promotion of transcriptional repression [70]	[71, 81]
	SUMO2/3	<i>in vitro</i>	n.r.	[82]
	SENP1 SUMO1/2 protease	n.r.	Enhancement of transcription at AR-driven genes	[86]

TABLE 1: Continued.

Phosphorylation				
	Kinase/ Phosphatase	Sites	Effects/biological relevance	Refs
HDAC2	SUMO1	K462 <i>in vivo</i> and <i>in vitro</i>	n.r.	unpublished data (C. V. Segre' and S. Chiocca)
Nitrosylation				
	Enzyme	Sites	Effects/biological relevance	Refs
HDAC1	n.r.	Tyrosine(s)	Response to cigarette smoke in macrophages	[96]
	n.r.	NOT s-nitrosylated	/	[98]
HDAC2	n.r.	Tyrosine(s)	Response to cigarette smoke in macrophages	[96]
	n.r.	Y68, Y73, Y167, Y146, Y173, Y253	Response to oxidative and nitric stress in bronchial cells	[73]
	n.r.	C262, C274 (s-nitrosylation)	Signal for proteasomal degradation (Y253)	
	n.r.		Response to BDNF-NO signalling in neurons	[97]
	n.r.		Promotion of dissociation from Fos, Egr1, Vgf, and Nos1 promoters	
	n.r.	s-nitrosylation	Partial rescue of myotube differentiation in Duchenne's cells	[98]
	n.r.	s-nitrosylation	Decrease of the enzymatic activity	
	n.r.	s-nitrosylation	Dissociation of the NuRD complex	[99]
	n.r.	s-nitrosylation	Activation of L1 β , TNF α , and MIP2 genes in macrophages	
Carbonylation				
	Enzyme	Sites	Effects/biological relevance	Refs
HDAC1	n.r.	C261, C273	Disruption of binding with transcriptional partners and histone substrates	[100]
			Transcriptional derepression of the HO-1, Gadd45, and HSP70 genes	
HDAC2	n.r.	carbonylated	n.r.	[100]

S: serine, Y: tyrosine, K: lysine, C: cysteine, n.r.: not reported in literature or not investigated, HAT: histone acetyltransferase, HDAC: histone deacetylase, E1/E2/E3: ubiquitin enzymatic cascade (see text).

RCS-induced inflammation) or to peculiar signaling pathway (such as the BDNF-NO axis). On the other hand, a broader spectrum of PTMs occurs on the C-terminal domains in response to a variety of different signaling pathways (such as hypoxia, hormone stimulation, extracellular signaling). This modular organization highlights the potential of this post-translational code for the modulation of HDAC1 and HDAC2 functions in many cellular pathways or biological responses to different stimuli.

As it was described in this paper, a broad number of single PTMs occur on HDAC1 and HDAC2 but strikingly very few examples of the interplay among them have been reported so far. The only reported example of PTM crosstalk occurs on HDAC2 in human bronchial epithelial cells when injured with cigarette smoke (CS) [55], (see Figure 2). In response to CS, HDAC2 associates with CKII, which in turn induces a strong phosphorylation of HDAC2 itself. CS also induces the formation of a complex between HDAC2 and the acetyltransferase CBP with consequent HDAC2 acetylation and increase in its transcriptional repressive activity. An HDAC2 quadruple mutant for all the CKII-dependent sites is far less acetylated than the wild type

in response to CS, showing that acetylation of HDAC2 relies on its CKII phosphorylation. Previous work from the same group [54] had also reported CKII dependent degradation of HDAC2 after CS exposure, and a global model was proposed. Cigarette smoke induces a bulk of phosphorylation of HDAC2, which determines its association with repressor partners and enhances its acetylation via CBP, contributing to further increase its repressive activities. On the other hand, CS-induced phosphorylation induces ubiquitination and degradation of HDAC2; reduction levels of HDAC2 are associated with steroid resistance in COPD and asthma [101], suggesting a mechanistic explanation for this resistance in smoker patients. It will be interesting in the future to verify which genes are repressed in bronchial cells in response to CS-induced phosphoacetylation of HDAC2 and if acetylation is linked to ubiquitination, or if they are two distinct parallel pathways driven by CKII phosphorylation.

Finally, oxidative and nitric stress induce nitrosylation on tyrosine 253 of HDAC2 in bronchial cells, which represents a signal for its proteasomal degradation [73]. Its possible link with CS-induced-CKII-phosphorylation and degradation has not been investigated. Nevertheless, it is clear that

cigarette smoke (and similar stress stimuli) evokes a complex PTM response on HDAC2, in which both the modular modification domains, the central and the C-terminal ones, are involved and cross interact. It is a step forward in the elucidation of the PTM code, but more investigation is still required to unveil the global panorama.

Another possible interplay between PTMs involves SUMOylation and CKII-phosphorylation of HDAC1. We have observed that the CKII phosphorylation mutant S421A, S423A is more widely SUMOylated than the wild-type HDAC1 [84]. This suggests a negative regulation of SUMOylation by the action of CKII, possibly as a consequence of the lost catalytic activity and binding properties of HDAC1 S421A and S423A, but the molecular mechanisms and the physiological role have not been investigated.

Finally, it was shown by two independent groups that ubiquitinated [79] and SUMOylated [89] forms of HDAC1 can be found at promoters of AR-driven genes and that both these modifications of HDAC1 contribute to the fine modulation of transcription. It will be challenging to investigate whether a crosstalk between SUMOylation and ubiquitination of HDAC1 exists at androgen-responsive promoters and to which extent this interplay affects gene transcription.

6. Conclusion: Why Deciphering the Code?

Class I histone deacetylases, and HDAC1 and HDAC2 in particular, have broad expression in many tissues and play a crucial role in cell cycle progression and proliferation [20]. HDAC1 and HDAC2 are deregulated in many cancers and are emerging as the main deacetylases involved in aberrant pathways of tumor cells in humans. Overexpression of HDAC1 is reported for gastric [102, 103], pancreatic [104, 105], colorectal [106], hepatocellular [107], and prostatic [108] carcinomas. HDAC2 plays a major role in APC-colon cancer; its overexpression is present already at the early polyp stage [109]. High levels of HDAC2 are found also in cervical dysplasia and invasive carcinomas [105]. High expression levels of both HDAC1 and HDAC2, together with HDAC3, have been associated with advanced stages of disease in prostate, gastric and colorectal cancers [105, 106].

Given the involvement of deacetylases in cancers, inhibitors of HDACs (HDACi) have been looked at as promising anticancer drugs and several classes of HDACi display potent anticancer activities *in vitro*. HDACi affect tumor cell growth and survival through induction of cell cycle arrest, block of angiogenesis, increase of antigenicity of tumor cells and induction of apoptosis [110]. A large number of clinical trials using different HDACi have shown their promising antitumor response *in vivo*; two of them have been approved by US FDA for the treatment of T-cell cutaneous lymphoma; suberoylanilide hydroxamic acid (SAHA, brand name vorinostat) in 2006 [111] and romidepsin (brand name istodax) in 2009 [112].

Unfortunately, their use is still restricted to specific types of cancer and adverse side effects have also been recorded, in particular to the blood system [113]. One of the main

reasons may be the fact that HDACi are not HDAC-isoform specific and this, given the differentiation of roles of single HDACs in different normal and cancer tissues, may represent a considerable limitation to their use in clinical settings.

HDAC1 and HDAC2 are not only relevant in cancer etiology, but are also important in other human diseases. An abnormal transcriptional repression of the growth factor BDNF by HDAC1/HDAC2 containing complexes is found in cortical neurons expressing the mutant huntingtin protein typical of the neurodegenerative Huntington disease [114]. HDAC2 was recently shown to be a crucial regulator of aberrant genetic programs in renal fibrosis associated with diabetes [115]. Finally, HDAC2 is involved in lung diseases such as COPD (as discussed above) and asthma [116].

It is clear that the integration of all the PTMs in a coherent and global code is a priority for understanding the regulatory networks operating on these key enzymes in different cellular, developmental, physiologic, and pathologic phenomena, with relevant clinical implications for a variety of human diseases.

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Review Article

Manipulating Protein Acetylation in Breast Cancer: A Promising Approach in Combination with Hormonal Therapies?

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Estrogens play an essential role in the normal physiology of the breast as well as in mammary tumorigenesis. Their effects are mediated by two nuclear estrogen receptors, ER α and β , which regulate transcription of specific genes by interacting with multiprotein complexes, including histone deacetylases (HDACs). During the past few years, HDACs have raised great interest as therapeutic targets in the field of cancer therapy. In breast cancer, several experimental arguments suggest that HDACs are involved at multiple levels in mammary tumorigenesis: their expression is deregulated in breast tumors; they interfere with ER signaling in intricate ways, restoring hormone sensitivity in models of estrogen resistance, and they clinically represent new potential targets for HDACs inhibitors (HDIs) in combination with hormonal therapies. In this paper, we will describe these different aspects and underline the clinical interest of HDIs in the context of breast cancer resistance to hormone therapies (HTs).

1. Introduction

1.1. Breast Cancer and Hormonal Therapies. Breast cancer is the most common malignancy and the second most common cause of cancer-related death amongst women in France, Western Europe and North America. About 70% to 80% of infiltrating breast carcinoma are estrogen receptor alpha (ER α) positive, thus offering clinicians the opportunity of hormonal therapies (HTs) in adjuvant and/or metastatic situation. Modulation of estrogen signaling pathways using antiestrogens (such as Tamoxifen or Fulvestrant) or more recently aromatase inhibitors (such as Exemestane, Letrozole, or Anastrozole) was indeed one of the first recognized targeted therapies and is currently the first-line treatment for ER α positive tumors [1]. The effectiveness of HTs is directly linked to the expression and functionality of ER α . Several retrospective studies and clinical trials have demonstrated that tumors expressing both ER α and progesterone receptor (PR) respond significantly better to HTs than those with

low receptor expression [2, 3]. Among patients who have a tumor expressing both ER α and PR, a benefit from HTs is seen in about 60% of cases, but the initial response is often not durable, since tumors become resistant to hormonal manipulation, leading to an “endocrine-resistant disease”. Moreover, patients with breast carcinoma lacking ER α (ER α negative) will not benefit from these therapies, as the expected efficiency of HTs in this situation is less than 10%.

Definition of the specific genetic lesions and molecular processes that determine clinical endocrine resistance is still incomplete. Candidate molecular pathways of intrinsic and acquired resistance to HTs emphasize the importance of signaling networks which control cell proliferation (e.g., acting *via* epidermal growth factor receptor type 2 (HER2) or insulin-like growth factor-1 receptor (IGF-1R)) or survival (through molecules such as Bad or Bcl-2) [4, 5]. In addition, polymorphisms in metabolizing enzymes such as the hepatic drug-metabolizing cytochrome P450 2D6 (CYP2D6) may

reduce the therapeutic benefit from tamoxifen (for a review, see [6]). Today, the main challenges in mammary cancer research are thus the development of more specific biomarkers to predict response or resistance to hormonal therapy and the development of new combined targeted therapies of hormone therapy-insensitive or therapy-resistant tumors.

1.2. Nuclear ER Signaling. Estrogens, like many other hormones, elicit numerous biological responses. They play a major role in the development and maintenance of the female reproductive tract (including the mammary glands) and are also involved in breast tumorigenesis. They act on target tissues through binding to two ER isoforms (ER α and ER β), which are members of the nuclear hormone receptor (NR) superfamily [7]. Upon interaction with ERs, estrogens induce a conformational change, which favors receptor dimerization and recruitment to promoter elements either directly through their DNA-binding domain or indirectly through interaction with other transcription factors. ER complexes then recruit transcriptional coregulators (coactivators and corepressors) to increase or inhibit target gene transcription [7]. In most cases, transcriptional cofactors are recruited as multiprotein complexes that could act either sequentially or simultaneously, depending on the considered gene. Many transcriptional coregulators of NRs exhibit enzymatic activities that participate in their mechanism of action. For example, several coactivators—CBP/p300, pCAF, SRC-1, and SRC-3—are acetyltransferases that are able to modify various lysine residues located in the amino terminal tails of histones. Conversely, inhibitory complexes associated with corepressors, contain histone deacetylases (HDACs) whose activity counteracts that of acetyltransferases (HATs). Some other enzymatic activities—including kinases or methyltransferases—displayed by coregulators are also able to modify histone lysines, arginines, or serines. All these posttranslational modifications interfere with each other and represent signals that enable binding of proteins involved in the transcriptional control of gene expression. From a clinical point of view, transcription therapies targeting pathological epigenetic modifications are very promising approaches to improve cancer treatment (see below).

2. Histone Deacetylases and Inhibitors

2.1. Acetylation of Chromatin and Nonchromatin Proteins. Acetylation and deacetylation of the ϵ -amino group of Lys residue (N $^{\epsilon}$) is a reversible reaction catalysed by the opposing actions of Lys acetyltransferases and Lys deacetylases. This modification, also described in bacteria, has been first extensively studied in the context of chromatin and histone modifications. As noted above, acetylation and deacetylation of the N-terminal tails of histones contribute to the “histone code” which defines part of the epigenetic landscape involved in the regulation of gene expression. It is now known that in addition to histones and transcription factors, N $^{\epsilon}$ -acetylation target numerous other proteins, such as proteins involved in cell signaling, DNA repair, metabolism, apoptosis, cytoskeleton, and protein folding (see also Section 5 of this paper).

N acetyl Lysine may serve as a docking structure for bromodomain, a protein domain that has the ability to recognize acetyl-lysine motifs. N $^{\epsilon}$ -acetylation may either enhance or decrease the function of the protein targeted, depending on the presence of other posttranslational modifications on the protein (such as phosphorylation and methylation) and the effects of N $^{\epsilon}$ -acetylation on protein/protein interactions [8].

Although both enzymes are involved in the modulation of protein acetylation, HDACs have been extensively studied as therapeutic targets, in particular in the context of cancer, while few studies have been performed on the clinical benefits of regulating HATs.

2.2. The HDAC Family. Up to now, eighteen human HDACs have been identified. They are divided into 4 families according to sequence homologies: class I (HDAC1, -2, -3, and -8) and class II (HDAC4, -5, -6, -7, -9, and -10) are homologous to the yeast histone deacetylases Rpd3 and Hda1, respectively, and share some degree of sequence homology. Class IV HDAC11 has been discovered more recently and shows similarities to both yeast Rpd3 and Hda1. Class I, II, and IV enzymes present a zinc ion-dependent catalytic domain. By contrast, class III enzymes (called sirtuins) are homologous to the yeast protein Sir2 and use NAD as a cofactor [9].

In the past few years, the crystal structure of the catalytic domain of human class I HDAC8 and class II HDAC4 and HDAC7 has been elucidated and several knockout mice targeting various HDACs have been generated, thus providing insights into their structure and physiological functions [10–13]. The diversity of HDACs suggests differential roles for the various classes of enzymes depending on tissues or cell lines. Accordingly, HDACs have been linked to cell cycle and proliferation and to the differentiation of various tissues. In addition to these physiological roles, the HDAC family has been involved in the physiopathology of human diseases including cancer. Fusion proteins containing HDACs complexes as well as deregulation of protein acetylation and/or HDACs expression have indeed been shown for various hematopoietic or solid tumors [14, 15]. Such findings have long encouraged the development of HDAC inhibitors as anticancer agents.

2.3. HDAC Inhibitors. Sodium butyrate (NaBu) was the first HDAC inhibitor (HDI) to be discovered in the late seventies, being initially found to have antitumor activity by inducing cell differentiation. Since then, various HDIs with different structures and potencies have been synthesized or purified from natural sources, and their effects as anticancer drugs are now widely documented. In 2006, suberoylanilide hydroxamic acid (SAHA or Vorinostat) was the first HDI approved by the FDA for the treatment of cutaneous T-cell lymphoma [16]. Today, the development of HDIs for the treatment of cancer is still ongoing and 80 phases I and II clinical trials are currently underway to validate these drugs alone or in association with other therapies in patients with hematological or solid tumors (see Section 6) [17, 18].

Different studies using cDNA array approaches have shown that around 10% of genes are modulated by HDIs,

with differences in the genes altered linked to the cell model, the time of culture, the concentration, and the HDIs used [19, 20]. Nevertheless, HDIs have been shown to have potent antitumor effects *in vitro* and *in vivo* on various cancer types affecting tumor cells at multiple levels: induction of cell cycle arrest, apoptosis and differentiation, inhibition of angiogenesis, inhibition of cell migration and invasion, and increase in antitumor immunity, response to radio- and chemotherapies (for reviews see [14, 21, 22]).

One of the challenges for the next years will be the development of more selective HDIs that would target specific HDAC isoforms to offer the patients the best therapeutic responses with the lowest toxicity. Specific HDIs have thus been described targeting class I HDACs and class II HDACs or HDAC8, some of them being tested in clinical trials, such as class I-specific MGCD0103 (Mocetinostat) in Hodgkin lymphoma [23]. Another challenge will be to search for biomarkers of clinical response to HDIs [24]. Some biomarkers have already been proposed such as histone H3 and H4 acetylation in tissues or peripheral blood mononuclear cells, HDAC2 tissue expression [25], gene expression profiles [26], or more recently expression of HR23B, a protein involved in the targeting of ubiquitinated proteins to the proteasome [27]. Despite encouraging results, the identification of potential biomarkers of response to HDIs is critically needed for future trials that will combine these drugs with endocrine therapy.

3. HDACs and Breast Cancer

3.1. HDAC Expression in Breast Cancers. HDAC expression in breast tumors has not been described for all members of the HDAC family, but mostly concerns class I HDAC1, -2, and -3 and class IIb HDAC6 at the protein and/or mRNA levels. Analysis of their prognostic significance in breast carcinoma has been performed in some studies (see below and [15] for a review).

Regarding mammary tumor progression, Suzuki et al. [28] reported a marked reduction in histone acetylation from normal mammary epithelium to ductal carcinoma *in situ* (DCIS) whereas most cases showed similar levels of acetylation in DCIS as compared to invasive ductal carcinoma. This suggests that alterations of histone acetylation are an early event in breast tumor progression. The authors also described a significant but smaller decrease in HDAC1, HDAC2, and HDAC6 protein levels during tumor progression. Greater reductions in HDAC1 protein levels were observed from normal to DCIS in estrogen-receptor negative and high-grade breast tumors (Table 1). According to the authors, such discrepancy (i.e., concomitant decrease in HDAC expression and histone acetylation) could be linked to the relative activities of both HATs and HDACs, as altered expression of HATs has been described in various cancers. It is also possible that the expression of other HDACs, not analyzed in this study, is increased during breast cancer progression, thus encountering for the global reduction in histone acetylation.

Analyzing invasive breast carcinoma, Krusche et al. detected HDAC1 protein expression in the nucleus of

TABLE 1: Expression of HDACs in relation with ER.

HDAC	HDAC expression	References
HDAC1	Reduced expression from normal to DCIS (ER- tumors)	[28]
	Correlation with ER expression	[29]
	High level of mRNA in ER+ breast cancers	[30]
HDAC2	Locus deletion in ER+ PR+ breast cancers	[31]
	Underexpressed in ER+ breast cancers	[32–55]*
HDAC3	Overexpressed in ER+ breast cancers	[44]*
	Correlation with ER expression	[29]
	Underexpressed in ER+ breast cancer	[56]*
HDAC4	Overexpressed in ER+ breast cancers	[45, 47]*
	Underexpressed in ER+ breast cancers	[38, 43, 53, 54, 57]*
HDAC5	Overexpressed in ER+ breast cancers	[34, 37, 39, 51, 52, 54, 57, 58]*
HDAC6	Overexpressed in ER+ breast cancers	[45, 47, 51, 53]*
	High level of mRNA in ER+ breast cancer	[59]
	Increased expression in ER+ breast cancer	[60]
	Underexpressed in ER+ breast cancers	[32, 34, 37, 50–52]*
HDAC7	Overexpressed in ER+ breast cancers	[36–38, 40, 44, 45, 47, 53, 58, 61]*
HDAC8	Underexpressed in ER+ breast cancers	[45, 47, 56, 62]*
HDAC9	Underexpressed in ER+ breast cancers	[32, 34, 35, 37–40, 45, 47, 52–54, 58]*
HDAC10	Overexpressed in ER+ ductal breast cancer	[48]*
HDAC11	Overexpressed in ER+ breast cancers	[35, 37–40, 43, 45, 47, 51–53, 56, 58]*
SIRT1	Overexpressed in ER+ breast cancers	[38, 40, 46, 52, 53]*

References with * were obtained from the Oncomine database. Increased or decreased expression was considered statistically significant at $P < .05$.

mammary luminal epithelial cells, but not in basal cells, and observed the presence of nuclear HDAC1 and HDAC3 proteins in 40% and 44% of breast tumors, respectively. They also found that HDAC1 and 3 protein levels correlated significantly with estrogen and progesterone receptors expression and that HDAC1 was an independent prognostic marker of better disease-free survival (DFS), but not overall survival (OS) in patients with invasive breast carcinoma

[29]. Similarly, Zhang et al. analyzed HDAC1 mRNA levels in invasive breast tumors and showed that HDAC1 mRNA levels were elevated in ER and PR positive tumors. They also found that patients with breast tumors displaying high levels of HDAC1 mRNA levels tended to have a better prognosis; however, in this study, HDAC1 was not found to be an independent prognostic marker of either DFS or OS [59].

Several studies have focused on HDAC6 expression in breast carcinoma. The rationale for such studies relies on initial results showing that HDAC6 was as an estrogen-responsive gene identified by a microarray approach and that it could modulate mammary tumor cell motility *in vitro* [60, 63]. More recently, Lee et al. also showed that HDAC6 was required for anchorage-independent growth of breast tumor cells [64]. HDAC6 protein was detected in 65% [59] and 77% [60] of breast carcinoma, with a cytoplasmic localization of the protein in both studies. Higher levels of HDAC6 mRNA were found in small, low-grade and ER+, PR+ breast tumors, that is, tumors of better prognosis, but this result was not confirmed at the protein level [59]. When analyzing the different studies, the prognosis significance of HDAC6 expression in invasive breast carcinoma remains controversial [15]. For instance, Yoshida et al. found that high levels of HDAC6 correlated with a negative prognosis survival whereas Zhang et al. showed that high levels of HDAC6 mRNA and protein was linked to improved DFS but not OS [59, 65]. On the other hand, Saji et al. did not link HDAC6 expression to DFS or OS, but found increased expression of HDAC6 in a subgroup of ER-positive, tamoxifen-responsive breast carcinoma.

Fewer studies have been performed on HDAC2 in breast carcinoma although its expression is frequently altered in cancer [14, 15]. In a recent analysis of genetic alterations associated with breast cancer subtypes, Hu et al. found deletions/loss of the HDAC2 locus in ER-positive and PR-positive breast tumors, but no data on HDAC2 expression were presented in this study [31]. HDAC2 mutations resulting in loss of HDAC2 protein and resistance to apoptosis induced by HDIs have been described in colon cancer [66]. However, to our knowledge, no mutations in HDAC2 or any other HDACs have been described in breast cancer.

In addition to these published data, we have performed data mining on HDAC expression in breast cancer using the Oncomine database (Compendia Bioscience, Ann Arbor, MI, USA-www.oncomine.org/). As shown in Table 2, the expression of some HDACs appears to be deregulated in breast cancers as compared to normal breast tissues. This is particularly true for HDAC2 and HDAC11 (overexpressed in cancer) or HDAC4–6 and the class III enzyme SIRT1 (underexpressed in cancer). In addition, the same data mining approach reveals that the expression of HDAC3–7, 10, 11, and SIRT1 at the mRNA level is higher in ER-positive breast cancers (Table 1).

In conclusion, although careful analysis of their expression and consequences in breast cancer have not already been performed for all members of the HDAC family, several studies and Oncomine data analysis underline the potential role of HDAC deregulation in breast tumor progression.

TABLE 2: Expression of HDACs in breast cancers.

Enzyme (locus)	Total studies	Increased expression in BC	Decreased expression in BC
HDAC1 (1p34)	5	1	1
HDAC2 (6q21)	7	5	2
HDAC3 (5q31)	5	1	2
HDAC4 (2q37.3)	6	1	5
HDAC5 (17q21)	5	1	4
HDAC6 (Xp11.23)	8	0	3
HDAC7 (12q13.1)	5	0	1
HDAC8 (Xq13)	5	2	1
HDAC9 (7p21.1)	6	4	2
HDAC10 (22q13.31)	8	0	1
HDAC11 (3p25.1)	6	3	1
SIRT1 (10q21.3)	9	0	3

From Oncomine database (Compendia Bioscience, Ann Arbor, MI, USA-www.oncomine.org/). Differential expression in breast cancer (BC) versus normal breast tissue was considered significant at $P < .05$. Bold numbers correspond to the strongest deregulations.

3.2. Effects of HDI on Breast Cancers—Experimental Data.

In breast tumor models, HDIs have potent antiproliferative effects *in vitro* and *in vivo* and interfere with estrogen signaling regulating ER α and ER β expression and function (see Section 4 and 5).

Various HDI have been shown to inhibit the proliferation of breast tumor cell lines, as well as normal human breast epithelial cells with IC50 ranging from nM to few mM depending on the HDIs tested [67–69]. This antiproliferative effect was found to be more pronounced in ER positive breast tumor cells than in ER negative ones [70, 71]. In various tumor models, this effect was in part linked to the induction of the cell cycle inhibitor p21 by HDI [70, 72]. Interestingly, p21 gene was found to more sensitive to HDI in ER positive than in ER negative mammary tumor cells, which may explain the observed difference in inhibition of cell proliferation upon HDI treatment according to the ER status [70]. Moreover, HDI were found to decrease Cyclin D1 expression and stability in mammary tumor cells and to inhibit phosphorylation of the retinoblastoma protein [71, 73–76]. Depending on the cell model, modifications of other cell cycle regulators have also been described including p27 and cyclin B1 [76, 77]. Accordingly, HDI induce cell cycle blockade at the G0-G1 and/or the G2/M level [74, 76–79].

TABLE 3: HDACs and estrogen signaling.

HDAC	Effect on estrogen signaling	References
HDAC1	Recruited to the silenced ER α promoter	[80]
	Present on ER-target gene promoter region	[81]
	Knockdown reduces ER α levels	[82]
	Directly interact with ER α -suppresses ER α activity in 293T cells	[83]
HDAC2	Present on ER-target gene promoter region	[84]
	Knockdown reduces ER α levels	[82]
HDAC3	Present on ER-target gene promoter region	[81]
HDAC4	Present on ER-target gene promoter region	[85]
	Binds the N-terminal A/B domain of ER α	[86]
HDAC5	Repress ER α promoter via MEF2	[87]
	Directly interacts with ER α -Represses ER α activity	
	KO associated with upregulation of ER α signaling	
HDAC6	Knockdown reduces ER α levels	[82]
	Bind the AF2-domain of ER α	[88]
	Regulates ER α degradation via hsp90 acetylation	[89]
HDAC7	Present on the pS2 gene promoter region	[84]
	Represses ER α activity-Required for E2-dependent repression	[90]
HDAC9	Repress ER α promoter via MEF2	[87]
	Directly interacts with ER α -Represses ER α activity	
	KO associated with upregulation of ER α signaling	
SIRT1	Deacetylates ER α <i>in vitro</i>	
	Knockdown reduces ER α levels	[91]

Most of these studies have been performed using HDI of broad range specificity. Recently, Duong et al. showed that inhibition of class II HDACs, using specific chemical compounds, also led to inhibition of mammary tumor cells proliferation in a dose-dependent manner, with higher potency in ER-positive than in ER-negative cell lines. In this study, specific inhibition of class II HDACs induced p21 expression, leading a cell-cycle blockade at the G0-G1 level [74]. Thus, although class II HDACs have been linked to cell differentiation, they may also be involved in cell proliferation, at least in this tumor model.

In vitro, HDI were found to induce apoptosis in breast tumor cells expressing or not ER α [74, 77, 92]. Depending on the cell type and/or the HDI used, apoptosis was linked to activation of the intrinsic (mitochondrial) and/or the extrinsic pathway. Some studies have shown upregulation of the proapoptotic Bak and Bim members along with a downregulation of the antiapoptotic survivin, XIAP and Bcl2 proteins in breast tumor cells [79] whereas others have found strong upregulation of the death receptors upon HDI [92, 93]. In addition, HDIs can efficiently sensitize breast cancer cells to TRAIL-mediated death signaling *in vitro*

and in preclinical *in vivo* models [77, 79, 94–96] and can significantly increase the apoptotic effects of various drugs targeting breast tumors.

HDI are also involved in cell differentiation. For instance, Davis et al. showed that NaBu induced cell differentiation in normal breast epithelial cell line as well as in breast cancer cells as indicated by accumulation of lipid droplets [67]. Using valproic acid (VPA), Travaglini et al. confirmed this result by measuring milk lipid production in cell cultures and showed that this effect was independent of the mammary cells ER status [76].

The antiproliferative and proapoptotic effects of HDIs observed *in vitro* were confirmed in preclinical mice or rat breast cancer models [68, 95, 97, 98]. HDIs were indeed shown to have anti-tumor activity *in vivo*, alone or in combination with other therapies, by inhibiting tumor growth or inducing tumor regression depending on the models, and this was found for ER α -expressing [68] as well as ER α -negative [98] breast tumor models. Interestingly, Hirokawa et al. further showed that the class I-specific HDI FK228 (depsipeptide or Istodax) was able to inhibit the growth of tamoxifen-resistant MCF-7 xenografts in nude mice (see below clinical studies) [97]. More recently, Palmieri et al. found that Vorinostat prevented the development of brain metastasis using a preclinical model of triple-negative breast cancer [99].

Taken together, these preclinical studies indicate that HDI have anti-tumor effects in breast cancer, targeting ER α -positive and ER-negative cells as well as the most aggressive mammary tumor types (tamoxifen-resistant and triple-negative tumors).

4. Regulation of ER Expression by HDACs

During the last decade, several groups have investigated the mechanisms by which HDACs regulate ER expression in breast cancer cells. These studies, which mostly concern ER α , have highlighted the multiplicity of the regulations involved (see Table 3 and Figure 1).

4.1. Negative Regulation of ER α Expression. In several ER α -expressing human cancer cells from different origin (breast, endometrium, ovary...), treatment with HDAC inhibitors such as trichostatin A (TSA), Vorinostat, FR901228, HC-toxin, VPA, LBH589 (Panobinostat), or NaBu produced a marked decrease in ER α expression at the mRNA and protein levels, which is independent of the presence or absence of ER ligands [82, 100, 101]. The mechanisms of this effect seem to involve different types of regulation which take place both at the transcriptional and posttranscriptional levels.

4.1.1. At the Transcriptional Level. A first level of inhibition of ER α expression takes place at the transcriptional level. Indeed, several studies have reported a decrease in ER α mRNA accumulation upon treatment with various HDAC inhibitors [100–102]. Concomitant treatment by TSA and cycloheximide, a protein synthesis inhibitor, did not affect the observed repression of ER α mRNA accumulation, suggesting a direct role for HDAC activity in the maintenance of

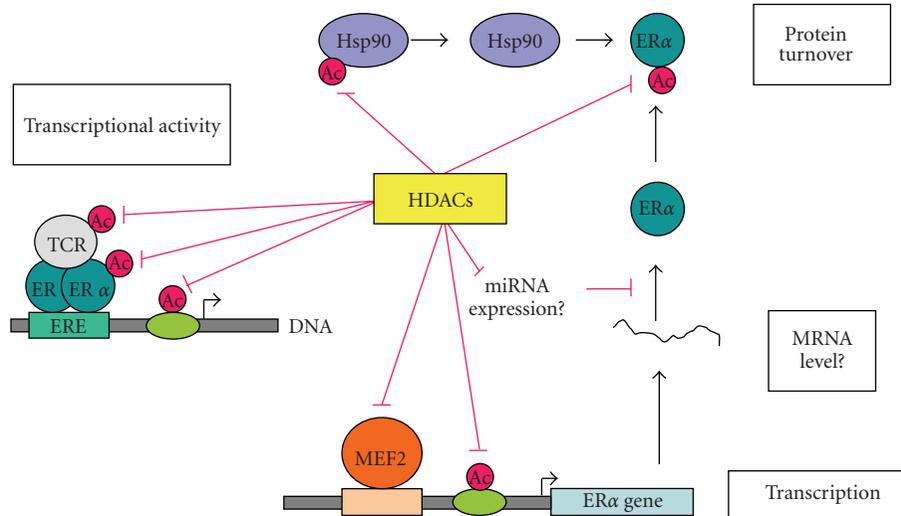


FIGURE 1: HDAC and estrogen signaling. HDACs are involved in estrogen-genomic mechanisms mediated in part through estrogen response element (ERE) targeting. ER α and numerous transcriptional coregulators (TCR) are acetylated proteins (acetyl mark is represented by a red circle) which are substrates for HDAC. By removing acetyl marks, HDAC regulate the transcriptional activity of ER α . HDACs also regulate the expression of ER α at the transcriptional level, in part through the control of MEF2 activity. They also modulate the level of ER α mRNA by a mechanism which might involve miRNA expression. Finally, HDACs also regulate ER α stability, and one mechanism appears to involve Hsp90 acetylation.

ER α transcription [73, 103]. Our unpublished data indicated that the stability of ER α mRNA was not significantly modified in ER α -expressing MCF7 cells treated with TSA.

Transcription of the ER α gene is driven by several different promoters which span over 300 kb (for a review, see [104]). In MCF7 and Ishikawa cells, levels of transcripts originating from promoters A, B, and C were all decreased upon TSA treatment. In endometrial cells, this effect was associated with a reduction of the amount of acetylated H3 and H4 on the three promoters confirming the inhibition of their activity [103].

Interestingly, both HDAC5 and HDAC9 (class II enzymes) have been shown to participate in the regulation of the ER α promoter by repressing the activity of MEF2 [87]. A recent study also reported that inhibition of SIRT1 by sirtinol or invalidation of the *SIRT1* gene was associated with a decrease of ER α expression in mammary cells which was the consequence of a transcriptional regulation [91]. Finally, several other HDACs could be involved in this negative regulation since a reduction in ER α expression was observed upon depletion of either HDAC1, HDAC2, or HDAC6 by siRNA in T47D breast cancer cells [82]. In these cases, the levels of regulation remain to be defined.

4.1.2. Regulation of mRNA Stability. A regulation of ER α mRNA expression could also take place at the posttranscriptional level since it has been reported that TSA when administered in combination with 5-Aza2'-deoxycytidine (5-azadC or Dacogen) could decrease ER α mRNA stability through altered subcellular localization of the RNA-binding protein, HuR [105]. In addition, several miRNAs (miR-206 for instance) have recently been reported to target

ER α mRNA (for a review, see [106]), and some of these miRNAs could be HDI induced and involved in the decreased expression of ER α .

4.1.3. Regulation of Protein Stability. Several data support a regulation at the posttranslational level. Results showing that the MG132 inhibitor relieves the TSA-mediated decrease of ER α accumulation ([102] and R. Margueron, unpublished observations) provide evidence for a direct or indirect involvement of the proteasome system in this regulation.

At the molecular level, one mechanism could involve the Hsp90 chaperone complex which binds to and maintains ER α in a ligand-binding conformation [107] and whose inhibition results in ubiquitin-mediated degradation of ER α by the proteasome [108]. Indeed, the chaperone function of Hsp90 has been shown to depend on HDAC activity, and HDAC6-specific inhibition leads to hyperacetylation of Hsp90, decreases its association with ER α , and results in ER α ubiquitination and depletion [89]. Data reported by Yi et al. confirmed that inactivation of the heat shock protein-90 (Hsp90) is involved in Vorinostat-induced ER α degradation and that the ubiquitin ligase CHIP (C-terminal Hsc70 interacting protein) enhances Vorinostat-induced ER α degradation [109].

By contrast, a recent paper indicated that TSA-induced acetylation of ER α in T47D cells was accompanied by an increased stability of the ER α protein [110]. Interestingly, in this study, overexpression of p300 also induced acetylation and stability of ER α by blocking ubiquitination.

4.2. Reexpression of ER α in ER-Negative Cells. An interesting aspect concerning ER α expression and HDAC inhibition

deals with data obtained in ER α -negative human breast cancer cells. The group of N. E. Davidson initially reported that treatment of such cells by TSA [111] or VPA [112] could lead to a dose- and time-dependent reexpression of ER α mRNA. In addition, TSA [113] or Scriptaid (another hydroxamic acid with HDI activity) [114] could potentiate the effect of DNA methyltransferase inhibitors such as 5-azadC on the reexpression of the ER α protein.

In ER α -negative MDA-MB-231 cells, the silenced ER α promoter has a repressive chromatin structure associated with DNA-methyltransferase 1 (DNMT1), DNMT3b, HDAC1, and H3-K9 methylation [80, 115]. The molecular mechanisms by which HDI reactivated silenced ER α gene in MDA-MB-231 cells include chromatin structure reorganization: for example, TSA induces acetylated histone H3 and H4 but reduces HDAC1 and H3-K9 methylation at the ER α promoter [80]. Chromatin immunoprecipitation analysis showed that binding of TFAP2C to the ER α promoter was blocked in ER α -negative cells, but that treatment with 5-azadC/TSA enabled TFAP2C and polymerase II binding [116].

In the ER α -negative human breast cancer cell lines MDA-MB-231 and MDA-MB-435, treatment with Panobinostat at 100 nM for 24 hours restored ER α mRNA and protein expression without a concomitant demethylation of the ER α promoter CpG island [117]. Importantly, the expression of ER α mRNA was sustained at least 96 hours after withdrawal of Panobinostat treatment. The same laboratory reported that reexpression of ER α protein upon treatment with Vorinostat, another pan-HDI, was coupled with loss of EGFR in MDA-MB-231 cells, which overexpress EGFR [118].

4.3. Regulation of ER β Expression. Fewer studies have described the effects of HDACs or HDAC inhibition on ER β expression in cancer cells. Recent studies reported the upregulation of ER β expression in ovarian [119] and prostate cancer cells at the mRNA and protein levels [120].

In breast cancer cells, HDI have been shown to clearly increase ER β expression at least at the transcriptional levels in both ER α -negative [121] and ER α -positive cells [122]. Moreover, treatment with HDI was found to strongly enhance the transcriptional activity of ER β [121, 122]. According to Jang et al. [121], ER β induction upon HDI treatment could be involved in the sensitization of ER α -negative breast cancer cells to hormonal therapy (see below).

5. Roles of HDACs and HDIs in ER Signaling

In addition to their role in the regulation of ER expression, a large set of data also support a major role of HDACs in the control of transcriptional signaling by estrogens (Table 3 and Figure 1).

5.1. Several Components of the ER Signaling Pathway Are Acetylated Proteins. Several types of posttranslational modifications have been described as targeting nuclear receptor (for a review, see [123]) and could modify several parameters such as DNA-binding activity, interactions with positive or

negative transcriptional regulators, and stability or subcellular localization of the protein. It has also been shown that ER α as other nuclear receptors could be modified at the posttranslational level by addition of acetylated groups on lysine residues [124, 125]. The group of Pestell initially reported that ER α was acetylated *in vitro* by p300 on two lysine residues located in the hinge region of the protein [126]. Mutation of the two amino acids resulted in an enhancement of hormone sensitivity, suggesting that acetylation normally decreases ligand response. More recently, using a variety of biochemical and cell-based approaches, Kim et al. identified two other lysines within ER α (K266 and K268) as primary targets of NCOA2-dependent p300 acetylation [127]. In this study, acetylation of these residues increased DNA-binding activity of the receptor in gel shift assay and ligand-dependent transactivation in transient transfection experiments. It should be noted that K266/268 are not conserved in ER β , and until now, acetylation of this nuclear receptor has not been reported. Moreover, the specific deacetylases which remove these marks are still mostly unknown although preliminary data suggested that both the NAD⁺-dependent SIRT1 enzyme or TSA sensitive HDAC are able to deacetylate ER α *in vitro*.

In addition to nuclear receptors themselves, several other factors involved in estrogen signaling are acetyltransferase substrates. Indeed, several nuclear receptor coregulators such as ACTR/SRC3, SRC-1 and TIF2 [128], PGC1 α [129], RIP140/NRIP1 [130], or HDAC1 [131] are also modified by acetylation, and this highlights the complexity of the effects resulting from the modulation of the acetylation balance in response to HDAC inhibition (see below).

5.2. Direct and Indirect Recruitment of HDACs by ERs. Using the chromatin immunoprecipitation technique (ChIP), the presence of several HDACs has been detected on various ER-target promoters. For instance, both HDAC1 and HDAC7 are present on the *pS2* gene promoter region [84, 90]. In the presence of partial antiestrogens such as tamoxifen or raloxifen, HDAC2 and HDAC4 [85] or HDAC1 and HDAC3 [81] have been evidenced on the *pS2* promoter or on other estrogen target promoters such as the *c-myc* or *cathepsin D* genes.

Several studies have reported different modes of HDAC recruitment by ER α . A direct association of HDAC1 with the DNA binding and AF2 domains of ER α has been demonstrated both by GST pull down and coimmunoprecipitation [83]. A more recent study failed to confirm this result, but it described the *in vitro* interaction with class II HDAC5 and 9 [87]. Finally, HDAC4 was shown to bind the N-terminal A/B domain of ER α [86] and, more recently, the physical E2-dependent association of HDAC6 with the AF2-domain of ER α expressed as a fusion with a membrane targeting signal was reported [88].

In addition to direct association with the receptor, HDACs could also be indirectly recruited to target promoters. Indeed, a huge number of ER transcription coregulators which bind the receptor in the presence of agonists (for instance RIP140 [132–134], SHP [135], and REA [136]) or in the presence of antagonists (such as NCoR or SMRT

[137, 138]) are able to recruit different HDACs. This indirect recruitment of HDACs belonging to the different classes of enzymes also increases the complexity of the relationship between acetylation and estrogen signaling.

5.3. Role of HDACs in the Control of ER Transcriptional Activity. As already mentioned, HDACs not only regulate ER expression *via* intricate mechanisms but also participate in the formation of ER transcriptional complexes. Different approaches, based on the modulation of their enzymatic activity or their expression levels, have indeed investigated whether or not they actively participate in the regulation of estrogen transcriptional signaling.

5.3.1. Effect of HDAC Inhibitors on ER Transcriptional Activity. Using MCF-7 or HeLa cells transfected with an ERE-containing luciferase reporter plasmid, we showed that inhibition of HDAC activity increased transactivation of both ER α and ER β in the presence of agonist ligands [122]. In ER α -expressing cells, HDAC inhibitors also abolished the transrepression ability of partial antiestrogens and increased their agonist activity through a mechanism which requires the reduction of ER α expression [100]. This effect was not obtained with class II selective inhibitors [74].

5.3.2. Class I HDAC. Very few data are available concerning the role of class I HDACs in the control of ER activity. Kawai et al. suggested that HDAC1 overexpression in 293T cells suppresses the E2-dependent transcriptional activity of ER α [83]. However, it is difficult to ascertain that this effect was indeed due to a modulation of receptor transactivation and not a simple reflect of a strong decrease in receptor levels. Using a Knockdown strategy, it has been reported more recently that selective depletion of HDAC2 in T47D cells resulted in a decrease in PR levels but it is unclear whether this is due to a modulation of ER α activity [82].

5.3.3. Class II HDAC. Class II HDACs have also been demonstrated to act as important modulators of ER α activity. A recent paper from the Olson's laboratory has demonstrated a role for HDAC5 and 9 in cardioprotection mediated by ER α [87]. Upregulation of ER α signaling in female mice deleted for either HDAC5 or -9 dramatically diminishes cardiac dysfunction following myocardial infarction. This cardiac protection appears to be due, at least in part, to the induction of neoangiogenesis in the infarcted region via upregulation of the ER target gene Vascular Endothelial Growth Factor (VEGF). These findings reveal a key role for MEF2 and class II HDACs in the regulation of cardiac ER signaling and the mechanisms underlying the cardioprotective effects of estrogen. Accordingly, van Rooij et al. showed that HDAC5 and HDAC9 repressed estrogen-dependent transcriptional activation by ER α .

Another class II HDAC, HDAC7, seems to play a unique role in E2-dependent repression of gene expression [90]. Indeed, in transient transfection experiments, increasing concentrations of HDAC7 inhibited ER α activity in a dose-dependent manner although the catalytic activity of HDAC7

did not appear to be required. More interestingly, knockdown of HDAC7 using siRNA resulted in complete loss of E2 repression of different target genes such as RPRM, CXCR4, or NEDD9.

HDAC4 has also been shown to regulate transactivation by ER α in the presence of either estradiol or antiestrogens such as tamoxifen or raloxifen [86]. Overexpression or silencing of HDAC4 impacted (negatively or positively) ER α activity in a cell type-specific manner.

Finally, HDAC6 may also participates in rapid action of estrogens (the so-called nongenomic action of ER), since it has been proposed that upon estrogen stimulation, a complex containing ER α and HDAC6 is rapidly translocated at the membrane, where HDAC6 could functionally interact with the microtubule network and cause tubulin deacetylation [88]. However, analysis of E2-induced tubulin deacetylation remains to be analyzed in HDAC6 knockdown or knockout models.

5.3.4. Class III HDAC. A single study has investigated whether class III HDACs play a role in the regulation of ER α activity [91]. This work demonstrated that sirtinol, an inhibitor of the SIRT1 deacetylase activity, inhibited estrogen-dependent gene transcription in different breast cancer cell lines. This observation could be related to previous data showing that the loss of SIRT1 expression in female mice is associated with a defect in mammary gland development [139].

6. Effects of HDIs on HT Response

6.1. In Vitro Experiments

6.1.1. ER α -Positive Breast Tumors. As discussed briefly in Section 3, several HDIs have been shown to reverse acquired hormone resistance in ER α -positive breast cancer cells lines. For example, Hirokawa et al. showed that treatment of tamoxifen sensitive and insensitive MCF-7 cells with deipeptide not only inhibited tumor cells proliferation *in vitro* and *in vivo* but also abrogated tamoxifen-resistance. These data suggest that HDIs could be useful for the treatment of breast cancers which become resistant to currently used estrogen antagonists such as tamoxifen [97]. Moreover, Hodges-Gallagher et al. suggested that this resensitization upon HDI, was not limited to tamoxifen, but could also be observed with aromatase inhibitors [140]. The mechanisms by which HDIs may reverse acquired hormone resistance in ER α -positive breast tumor cells are probably complex and may involve different mechanisms according to tamoxifen or antiaromatase treatments. For instance, inhibition of HDAC enzymatic activity modulates ER α and ER β expression and may control the relative agonist activity of partial antiestrogens (see above) [100]. Moreover, HDIs block the activation of PAK1 [97], a growth factor pathway, which may contribute to tamoxifen resistance [141]. In addition, De Los Santos et al. showed that a combination of Vorinostat and fulvestrant (a pure steroidal anti-estrogen also known as ICI 182.780) was more potent than fulvestrant alone

to regulate the expression of cell cycle proteins, to induce downregulation of ER α , and to decrease the transcription of ER α target genes in MCF-7 breast cancer cells [75].

To our knowledge, no preclinical study has been published evaluating the effects of a treatment combining HDI and HTs (tamoxifen or aromatase inhibitor) on the delay of endocrine acquired resistance in ER α -positive breast cancer cells or in xenografts.

6.1.2. ER α -Negative Breast Tumors. As stated in Section 1, HTs are ineffective in ER α -negative breast carcinoma. Preclinical studies have shown that ER α repression in these tumors may be due to epigenetic modifications. The discovery of HDACs recruitment in ER α gene promoter provides a rationale for inhibiting HDACs activity to release ER α transcriptional repression as a potential therapeutic strategy (see Section 4). Several laboratories have reported that HDIs could reverse hormone resistance in human ER α -negative breast cancer cells. The combination of TSA and 5-azadC, a DNMT inhibitor, restored sensitivity to tamoxifen in MDA-MB 235 human breast cell lines and in nude mice. This effect was due to the reexpression of a functional ER α and the level of tamoxifen growth suppression paralleled that of ER α reexpression [98]. Similarly, restoration of ER α expression by the pan HDI Panobinostat in MDA-MB 231 cells enhanced sensitivity to 4-hydroxy-tamoxifen (an active metabolite of tamoxifen) [117]. So, reexpression of ER α might at least in part mediate the antiproliferative effect of tamoxifen, although other mechanisms are likely to be involved. For instance, Jang et al. observed that pretreatment of ER α -negative MDA-MB 231 and Hs578T breast cancer cells with TSA alone could restore response to tamoxifen whereas no apparent ER α could be detected in the treated cells. The mechanism involved might be linked to the upregulation of ER β expression [121]. Other mechanisms may involve modulation of growth signaling pathways. Zhou et al. indeed showed that Panobinostat allowed a decrease in EGFR expression together with the suppression of EGF-initiated signaling pathways involved in the loss of tamoxifen antiestrogenic effect including phosphorylated PAK1, p38MAPK, and AKT [142]. Treatment of ER α -negative and hormone resistant human breast cancer cells MDA-MB 231 or xenografts with the HDI SNDX275 (MS275 or Entinostat) led to an upregulation of ER α and aromatase expression. Importantly for clinical perspectives, these up regulations resulted in a sensitization of MDA-MB 231 cells and xenografts to a treatment with an aromatase inhibitor (Letrozole). The same authors reported inhibition of growth, cell migration, and formation of micrometastasis by treatment with Entinostat plus letrozole (Sabnis et al., communication at the San Antonio Breast Cancer Symposium 2009). Altogether, these results provide the basis of therapies combining tamoxifen (or aromatase inhibitors) and HDIs for the treatment of hormone refractory ER α -negative breast cancer and open a new perspective for the management of ER α -negative breast cancer.

6.2. Clinical Trials. Several HDIs have been used in clinical trials for the treatment of hematological malignancies (with

great success in most studies) and for solid tumors although with less impressive clinical efficacy. Concerning breast cancer, the HDIs Vorinostat, Panobinostat, and Entinostat are currently being tested in patients with advanced and/or metastatic disease. The most common adverse events of HDI treatment include fatigue, nausea, diarrhea, thrombocytopenia, and lymphopenia [17, 18]. In metastatic breast cancers, HDIs have limited efficacy as single agents. For example, a phase II study evaluating Vorinostat alone was stopped early due to the absence of objective responses [143]. A phase II study evaluating the efficacy of Panobinostat alone in HER2-negative women with locally recurrence or metastatic breast cancer is still ongoing (NCI clinical trial protocol NCT00777049; see <http://www.cancer.gov/>).

As stated above, HDIs as single agents have shown limited activity in patients with solid tumor malignancies, thus prompting clinicians to use these compounds in combination with other therapies acting on other targets than HDACs. Such drug combinations interfering with both HDACs and growth factor pathways (HER2, EGFR, BCR-ABL, etc.) have already shown promising anticancer effects *in vitro* [144–146]. Moreover, studies combining an HDI with chemotherapy (Munster et al., communication at the San Antonio Breast Cancer Symposium 2009) or trastuzumab, an HER2 monoclonal antibody, (NCI clinical trial NCT00567879) are ongoing. Preliminary results, in heavily pretreated women who had either relapsed or progressed during trastuzumab combined therapies proved to be promising: Vorinostat or Panobinostat were indeed shown to reverse trastuzumab resistance.

Since there is a good rationale for combining HDI with HTs, several trials involving the combination of a pan- or a selective HDI and an HT (tamoxifen, or aromatase inhibitor) are ongoing (see Table 4). At the SABCS 2009, Munster et al. reported preliminary results of a phase II study, combining Vorinostat (400 mg daily for 21 days of 28 days) and tamoxifen (20 mg daily), in women with ER-positive metastatic breast cancers whose tumor progressed under aromatase inhibitors treatment. Moreover, patients could have received up to 3 chemotherapy regimens for metastatic disease. In the first 42 patients enrolled in this trial, 34 were assessable for efficacy to the date of the report: 7 (21%) had an objective response, and 4 (12%) had stable disease for ≥ 6 months. These results are encouraging if one considers that the expected response rate for tamoxifen alone at this stage of disease is less than 10% and that the trial of Luu et al., evaluating Vorinostat alone in metastatic breast carcinoma reported no objective responses [143].

Moreover, a preliminary phase II data suggest that Entinostat, a class I selective HDI, may resensitize invasive ER-positive breast cancer patients progressing under aromatase inhibitors although 80% of these patients had already received tamoxifen (Yardley et al., communication at the San Antonio Breast Cancer Symposium 2009). In metastatic breast carcinoma, additional trials involving the combination of an HDI including Vorinostat and Panobinostat with aromatase inhibitors are underway.

TABLE 4: Clinical trials combining HDI and HT in advanced/metastatic ER-positive breast carcinoma.

HDI	HT	Phase	Patients	Preliminary results	Reference
Vorinostat (SAHA) 200 mg twice daily 14 d/21	Tam	II	AI resistant HR+	34 patients evaluated 21% OR 12% SD	Munster et al. Poster # 6100 SABCS 2009
Entinostat (SNDX275) 5 mg weekly	AI (Exemestane)	II	AI resistant HR+	10 patients with >2 cycles CB > 6 months (1 case) CB > 5 months (2 cases)	NCI clinical trial NCT00676663 Yardley et al. Poster # 6111 SABCS 2009
Vorinostat (SAHA) 200 mg twice daily 14 d/21	AI (anastrozole letrozole, OR exemestane)	II	AI resistant	Ongoing	NCI clinical trial NCT01153672 Linden et al.
Panobinostat (LBH589) once daily on days 1, 3, 5 during 28 d	AI (Letrozole)	I/II	HR-/+ (phase I) triple-negative disease (phase II)	Ongoing	NCI clinical trial NCT01105312 Tan et al.
Vorinostat (SAHA)	Tam	II	Stage I–III (treatment for 2 weeks before surgery)	Ongoing	NCI clinical trial NCT01194427 Stearns et al.

AI: aromatase inhibitor. OR: objective response. SD: stable disease. CB: clinical benefit. HR: hormone receptor. SABCS: San Antonio Breast Cancer Symposium 2009.

7. Perspectives and Conclusions

In conclusion, analysis of the links between ERs and HDACs underline multiple and intricate levels of interactions. Such complexity is reflected in breast tumorigenesis as HDI have opposite effects on ER α expression in ER-positive and ER-negative breast tumor cells. Several important questions remain to be answered in order to further appreciate these transcriptional and cellular crosstalks: what are the roles of the different HDAC isoforms? Do HDAC regulate ER signaling independently of their catalytic activity? Are sirtuins key players in these crosstalks? Do HDACs regulate miRNA which target ER signaling? What is the exact role of HDACs in hormone resistant breast tumors?

Despite these open questions, HDI in combination with chemotherapies or hormonal therapies led to promising results in the context of hormone-resistant breast cancers, and several clinical trials are still ongoing in this field. Further studies are needed to define the best combinations of HDI therapies for the most aggressive breast tumors and to better understand how they impact hormone-resistant breast cancers.

Moreover, as stated in this paper, much work is being done today to define biomarkers that would identify which tumors will better respond to HDI-combined treatments. In the field of breast cancer, it will certainly be important to define biomarkers for the reexpression of ER α in ER-negative tumors along with predictive biomarkers of anti-estrogen sensitivity in hormone resistant tumors in response to HDI treatments. Finally, few data have been performed on triple negative breast tumors, which represent one of the most aggressive groups of breast cancers or in the familial forms of

BRCA1 mutated tumors. The role of HDACs and the impact of HDIs in these particular groups could possibly open new therapeutic strategies.

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Review Article

Histone Deacetylase Inhibitors in the Treatment of Hematological Malignancies and Solid Tumors

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The human genome is epigenetically organized through a series of modifications to the histone proteins that interact with the DNA. In cancer, many of the proteins that regulate these modifications can be altered in both function and expression. One example of this is the family of histone deacetylases (HDACs), which as their name implies remove acetyl groups from the histone proteins, allowing for more condensed nucleosomal structure. HDACs have increased expression in cancer and are also believed to promote carcinogenesis through the acetylation and interaction with key transcriptional regulators. Given this, small molecule histone deacetylase inhibitors have been identified and developed, which not only inhibit HDACs, but can also lead to growth arrest, differentiation, and/or apoptosis in tumors both *in vitro* and *in vivo*. Here, we will discuss some of the recent developments in clinical trials utilizing HDACs inhibitors for the treatment of both hematological malignancies as well as solid tumors.

1. Introduction

DNA is woven together with proteins into an intricate organization of both extended euchromatin and condensed heterochromatin. The posttranslational modifications of the histone proteins involved in this structure regulate the epigenetic organization of the genome. This genomic organization is often altered on an epigenetic level, including the phosphorylation, acetylation, methylation, ubiquitination, sumoylation, and ADP-ribosylation of the eight histones within the nucleosome (H2A, H2B, H3, and H4).

In 1964, Mirsky and Allfrey published the first reports of histone acetylation and methylation being involved in RNA synthesis in a reversible fashion and being highly associated with open chromatin [1, 2]. Today, it is known that histone acetyltransferases transfer the acetyl group from acetyl-CoA forming ϵ -N-acetyl lysine on conserved lysines of the N-terminal tails of histones H3 and H4 (and to a lesser extent H2A and H2B), resulting in an open nucleosomal

structure. This can be reversed by histone deacetylases (HDACs) of which, in mammals, there are currently 18 identified and have been divided into four classes based on cellular localization and function [3]. Class I includes HDACs 1, 2, 3, and 8 which are all nuclear and ubiquitously expressed. Class II, being able to shuttle back and forth between the nucleus and the cytoplasm and believed to be tissue restricted, includes HDACs 4, 5, 6, 7, 9, and 10; within this class, HDACs 6 and 10 (class IIb) have two catalytic sites, are expressed only in the cytoplasm, and are involved in a variety of biological processes. Class III contains the structurally diverse NAD⁺-dependent sirtuin family, which does not act primarily on histones [4]. Finally, the ubiquitously expressed HDAC11 represents Class IV, which has previously been characterized as being part of both Class I and Class II (Figure 1). Nonhistone targets of HDACs include p53, E2F, GATA-1, YY1, RelA, Mad-Max, c-Myc, NF- κ B, HIF-1 α , Ku70, α -tubulin, STAT3, Hsp90, TFIIE, TFIIIF, and hormone receptors explaining the diverse biological

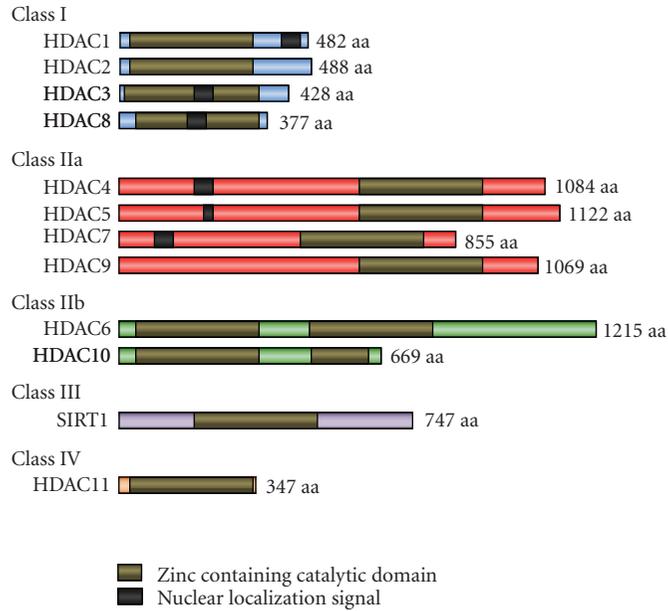


FIGURE 1: The histone deacetylase, family. Schematic representations of class I (HDAC1, 2, 3, and 8), class II (HDAC4, 5, 6, 7, 9, and 10), class III (SIRT1), and class IV (HDAC11). Structure and Length of HDACs are shown. The total number of amino acid residues (aa) is depicted on the right, next to each HDAC. The enzymatic domains and the nucleus localization sequences are highlighted in brown and black, respectively.

effects that HDACs can impart to the cell ([5–17] for review, see [18, 19]).

Knockout mice for HDACs 1 and 2 display embryonic or perinatal lethality and class II HDACs knockouts, while viable and fertile (except for HDAC7) have significant developmental abnormalities [20–22]. HDACs expression, and activity can be altered in many cancers and in both lymphoma and leukemia HDACs is associated with the function of oncogenic-translocation products, such as PML-RAR α in acute promyelocytic leukemia [23–25]. Furthermore, with the discovery of specific pan-HDACs inhibitors, it has been shown that blocking HDACs function can cause cell-cycle arrest and differentiation through the increased expression of p21^{WAF1/CIP1} [26, 27], affect tumor survival by blocking angiogenesis through the increased acetylation of HIF-1 α [9], affect protein degradation through the acetylation of Hsp90 [13], and increase the expression of pro-apoptotic factors [28–31], making HDACs inhibitors a good candidate for single-agent cancer therapy and even combination therapy with conventional chemotherapeutics and radiation. Here, we will discuss the latest clinical advances in HDACs inhibitors.

2. HDACs Inhibitor Classifications

Riggs and colleagues identified the HDACs inhibitor prototype sodium butyrate to be an effective inhibitor of deacetylase activity [32, 33]. This was found to be non-competitive, reversible and specific for HDACs activity [34–36]. Sodium butyrate was also found to induce differentiation, RNA synthesis and strongly inhibit cell growth in the G1 phase of the cell cycle [37]. These findings paved

the road for development of more specific and effective HDACs inhibitors to use in the clinic. HDACs inhibitors can be divided into four major structural classes: (1) small molecular weight carboxylates; (2) hydroxamic acids; (3) benzamides; and (4) cyclic peptides [19, 38, 39]. Pan-HDACs inhibitors include vorinostat, panobinostat, belinostat and isotype/class-specific HDACs inhibitors include romidepsin, meletinostat (MGCD0103) and entinostat [39]. Vorinostat (Zolinza) and Romidepsin (Istodax) are the only HDACs inhibitors currently approved by the U.S. Food and Drug Administration (FDA) for the treatment of refractory cutaneous T-cell lymphoma (CTCL) [40, 41].

All HDACs inhibitors available or in development target the zinc molecule found in the active site of Class I, II, and IV HDACs and are characterized by their ability to inhibit the proliferation of transformed cells in culture and tumor growth in animal models by inducing cell-cycle arrest, differentiation, and/or apoptosis (Figure 2). It has been shown that HDACs inhibitors can selectively induce the expression of less than 10% of genes, some of which are involved in the inhibition of tumor growth (e.g., p21^{WAF1}, p27^{Kip} and p16^{ink4a}) [19, 26, 38]. Furthermore, evidence shows that more genes may be repressed after HDACs inhibitors treatment than activated, this could be due to a chromatin conformation in a hyperacetylated state that represses transcription, the release of transcriptional repressors from HDACs protein complexes, the activation or inactivation of nonhistone transcriptional repressors and many other plausible explanations. Unfortunately, the mechanism of action is not completely elucidated, and there are also no substantiated HDAC or HAT measurements that can predict tumor response to HDACs inhibitors treatment.

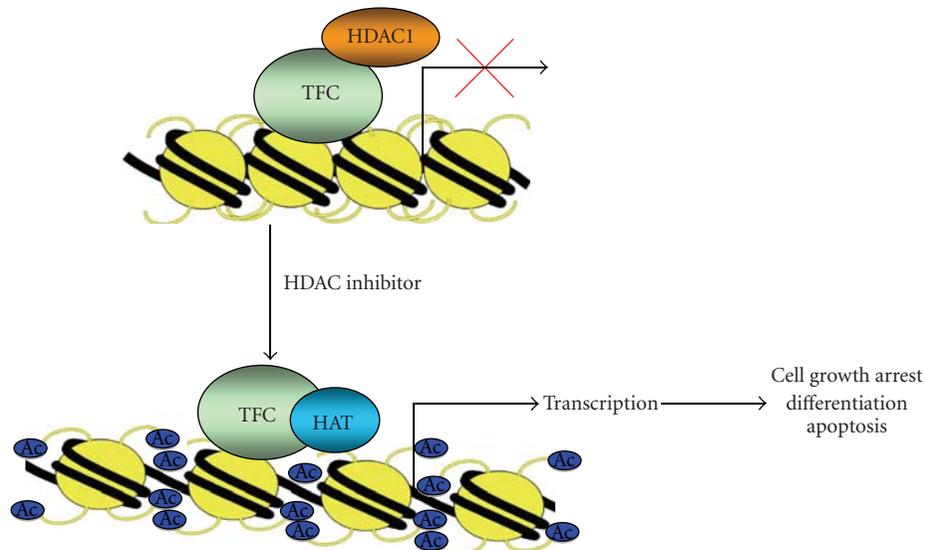


FIGURE 2: Mechanism of action of histone deacetylase inhibitors. It has been proposed that there are specific sites in the promoter region of a subset of genes that recruit the transcription factor complex (TFC) with histone deacetylases (HDACs). With inhibition of HDACs by HDACs inhibitors, histones are acetylated, and the DNA that is tightly wrapped around a deacetylated histone core relaxes. The accumulation of acetylated histones in nucleosomes leads to increased transcription of this subset of genes, which, in turn leads to downstream effects that result in cell-growth arrest, differentiation, and/or apoptosis.

Otherwise, HDACs inhibitors induce broad hyperacetylation in both tumor and normal tissues, which can be used as a biomarker for drug activity. However, steps will need to be taken to further characterize the molecular mechanisms behind HDACs inhibitors function as well as predictive markers of response to further implement them functionally in the clinic.

3. HDACs Inhibitors in Clinical Trials

From the initial discovery of sodium butyrate, there has been tremendous interest and investigation in HDACs inhibitors, today there are at least 15 HDACs inhibitors that are currently under clinical investigation for both hematological malignancies and solid tumors, both for single-agent and combination therapy [42]. Initial molecules included valproic acid, phenyl-butyrate, SAHA (vorinostat), trapoxin A, oxamflatin, depudepsin, depsipeptide (romidepsin, Istodax) and trichostatin A [38, 43], which have paved the way to the second-generation HDACs inhibitors such as the hydroxamic acids: belinostat (PDX101), LAQ824, and panobinostat (LBH589), and the benzamides: entinostat (MS-27-275), CI994, and MGCD0103 (mocetinostat) [44]. Here, we will discuss some of the recent clinical trials regarding several of the most promising HDACs inhibitors (Table 1).

4. Vorinostat

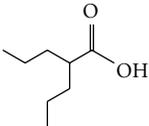
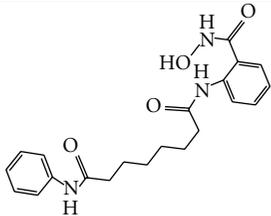
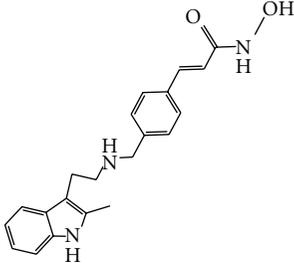
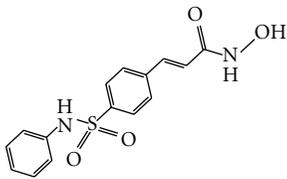
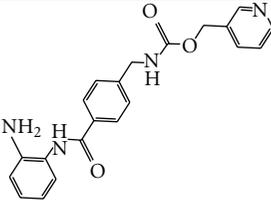
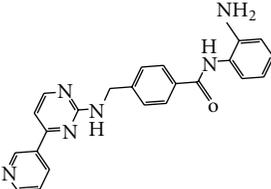
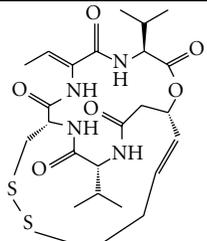
In 2006, two phase II trials led vorinostat (Zolinza) to be approved by the U.S. FDA for the treatment of refractory cutaneous T-cell lymphoma CTCL [40]. A multicenter phase IIB trial enrolled a total of 74 patients for progressive,

persistent, or recurrent CTCL who had received at least two prior therapies. Patients were treated daily with 400 mg of orally administered vorinostat and showed an overall response rate of 29.7%, a 6.1 month median duration of response, and a 9.8 month median time to progression [45]. Similar findings were published in a phase II study with a similar patient population [46]. When considering all patients from these trials together, 26% of patients experienced thrombocytopenia, 14% anemia, and only 5% of patients experienced grade 3 to 5 adverse events, including thrombocytopenia, pulmonary embolism, fatigue, and nausea. The most common adverse events were diarrhea, fatigue, and nausea. From the larger multicenter trial, 6 patients continued treatment with vorinostat for 2 years or longer with continued clinical effect (one complete remission (CR), four partial remission (PR), and one stable disease (SD)) [47].

A phase II clinical trial tested the use of vorinostat in other hematological malignancies, including relapsed diffuse large B-cell lymphoma (DLBCL), where out of 18 patients, one resulted in a CR and one in SD with grade 1 and 2 toxicities, but was concluded to have an overall minimal effect in treating DLBCL [48]. A second trial tested vorinostat in patients with lymphoma showing promising results. Out of 17 patients with relapsed indolent non-Hodgkin's lymphoma four patients achieved CR, two had PRs and four patients remained with SD [49].

A dose-escalation phase I trial was also performed for oral vorinostat as a single-agent therapy in acute myeloid leukemia (AML). Out of 41 total patients enrolled, 31 with AML, three with myelodysplastic syndrome (MDS), four with chronic lymphocytic leukemia (CLL), two with acute lymphoblastic leukemia, and one with chronic myeloid

TABLE 1: Table of HDACs inhibitors discussed in this paper, organized by class (refer to text for references).

HDACs inhibitor class	HDACs inhibitors	Other common identifiers	Clinical trial phase	Structure
Small molecular weight carboxylates	Valproic acid	Depakene, Depakote, Depakote ER, Depakote Sprinkle	FDA-approved for epileps, seizures, mania, bipolar disorders, migranes Phase I/II in hematological malignancies and solid tumors	
Hydroxamic Acids	Vorinostat	Suberoylanilide hydroxamic acid, (SAHA), Zolinza	FDA-approved for CTCL Phase I/II in hematological malignancies and solid tumors	
	Panobinostat	LBH589	Phase I/II in hematological malignancies and solid tumors	
	Belinostat	PXD101	Phase I/II in hematological malignancies and solid tumors	
Benzamides	Entinostat	MS-27-275, MS-275, SNDX-275	Phase I/II in hematological malignancies and solid tumors	
	MGD0103	Mocetinostat	Phase I/II in hematological malignancies and solid tumors	
Cyclic tetrapeptides	Romidepsin	Depsipeptide, Istodax, FK228, FR901228	FDA-approved for CTCL Phase I/II in hematological malignancies and solid tumors	

leukemia (CML). The maximum tolerated dose (MTD) was 200 mg when given twice daily and 250 mg when given three times daily, each given for 14 days in a 21-day cycle. The dose limiting toxicities (DLT) were again nausea, vomiting, and diarrhea. Seven of the patients with AML showed hematologic responses, including two CRs and two CRs with incomplete recovery [50].

Vorinostat has also been tested for use in treating several solid tumors, including platinum-resistant epithelial ovarian cancer, primary peritoneal carcinoma, and nonsmall cell lung carcinoma (NSCLC). After encouraging results from a phase I dose-escalation trial of vorinostat combined with carboplatin and paclitaxel in advanced solid malignancies, resulting in 11 out of 25 patients (10 of 19 with NSCLC and 1 of 4 with head and neck cancer) achieving a PR [51], a phase II National Cancer Institute-sponsored study has been carried out and results recently published [52]. This phase II randomized, double-blinded, placebo-controlled trial enrolled 94 patients with previously untreated stage IIIB or IV NSCLC to receive Carboplatin and Paclitaxel with either Vorinostat (400 mg daily on days 1 through 14 of each treatment cycle) or placebo. In the Vorinostat arm, a favorable trend toward improvement in median PFS (6 months versus 4,1 months in the placebo arm) and OS (13 months in the Vorinostat arm versus 9,7 months in the placebo arm) was clearly shown although at the price of an increased toxicity. Grade 4 thrombocytopenia was more frequent in the Vorinostat arm (18% versus 3% in the placebo arm) as well as grade 2-3 nausea, diarrhea, and fatigue. Moreover, 26% of patients in the Vorinostat arm discontinued therapy after the first cycle in comparison to 16% of the ones enrolled in the placebo arm. Comparably, the proportions of patients who completed all 6 cycles scheduled were 41% and 29%, respectively, for the placebo and Vorinostat arm.

Several trials also tested the efficacy of Vorinostat as single agent in different solid tumor sites (head and neck, breast, colorectal, and prostate cancer) and all reported a considerably high rate of adverse effects limiting the possibility of a reliable efficacy assessment. The most common adverse event reported in those trials were: fatigue (from 62% to 81%), nausea (from 58% to 74%), anorexia (from 58% to 81%), vomiting (from 33% to 56%), and thrombocytopenia (from 17% to 50%) [53–55].

Vorinostat is potentially also an attractive candidate for association with radiation since HDACs inhibition decreases cellular ability to repair DNA double-strand breaks both by Homologous Repair (HR) and Non-Homologous End Joining (NHEJ) [56, 57], thus resulting in a potent *in vivo* radiosensitizing effect [58]. A Phase I trial recently tested Vorinostat in combination with pelvic palliative radiotherapy (3 Gy per fraction up to 30 Gy) for gastrointestinal tumors. Vorinostat was administered orally once daily 3 hours before each radiotherapy fraction at doses ranging from 100 mg to 400 mg. The most common, any grade, adverse effects reported were fatigue, nausea, anorexia, and vomiting, respectively, in 94%, 65%, 59%, and 47% of patients [59].

5. Romidepsin

Romidepsin (Istodax, Gloucester Pharmaceuticals) is a natural compound isolated from *Chromobacterium violaceum*. It is a bicyclic tetrapeptide and is sometimes referred to as depsipeptide after the class of molecules to which it belongs. It was first tested for antibacterial activity, but it was found to have strong cytotoxic activity against different tumor cell lines, and later on mice. Romidepsin is mainly targeting class I HDACs, and it has also been recently approved by the FDA for treatment of CTCL. Two phase II multicentric single arm trials collected cumulatively 167 patients with refractory CTCL (mostly in advanced stages) treated with Romidepsin at a starting dose of 14 mg/m² infused over 4 hours on days 1, 8, and 15 every 28 days. The endpoint for both studies was the overall response rate (ORR). Median time to first response was 2 months in both studies and ORR was 34% and 35%, respectively. The median duration of response was 15 and 13.7 months, respectively. Adverse effects observed in both studies were similar to the toxicities observed in phase I trials. Common (any grade) adverse effects included nausea (56% and 86%, resp.), fatigue (53% and 77%), vomiting (34% and 52%), and anorexia (23% and 54%). Furthermore, consistently with the toxicity pattern shown by Romidepsin in Phase I studies [60], ECG changes were also noted in a large proportion of patients of the study (up to 50%) consisting of T-wave flattening, ST tract depression, and QT interval prolongation. Cardiotoxicity, which has not been frequently found after Vorinostat treatments, seems to be a more specific side effect of Romidepsin and has been explained as being dependent upon the interaction of the drug with the HERG K⁺ channels [3]. Romidepsin has also been initially tested clinical conditions other than CTCL. In some Phase I/II trials, single-agent Depsipeptide has shown a limited clinical benefit in treating refractory neoplasms, including AML/MDS, CLL, lung cancer, hormone refractory prostate cancer, and renal cell cancer [61–66].

6. Mecetinostat (MGCD0103)

Mecetinostat (MGCD0103) is a class I isotype-selective orally available benzamide HDACs inhibitor. Early clinical trials have demonstrated activity in hematological malignancies, including myeloid leukemia and lymphoma and was well tolerated with DLTs of fatigue, nausea, vomiting, and diarrhea. A phase I trial resulted in a bone marrow CR in three of 29 patients with AML at a MTD of 60 mg/m² administered three times weekly [67]. A phase II study in adults with relapsed or refractory DLBCL (33 patients) or follicular lymphoma (FL—17 patients) also demonstrated significant anticancer activity. Most of the 17 patients with DLBCL that were reassessed by CT after treatment showed a decrease in tumor volume, as well as one CR and 3 PRs. Out of ten patients with FL, one achieved PR. Grade 3 toxicities or greater included fatigue, neutropenia, thrombocytopenia, and anemia [68]. A phase II trial was also conducted in patients with relapsed or refractory Hodgkin's lymphoma. A treatment schedule of 110 mg or 85 mg three times per week in a 4-week cycle were given to 23 and 10 patients, respectively. From the 21 patients

evaluated from the 110 mg cohort, there was an ORR of 38% (2 had CRs, and 6 had PRs). The patients who had CRs remained with progression free survival for >270 and >420 days, respectively. From the 10 patients in the 85 mg cohort, all 5 that were evaluated demonstrated tumor reductions of $\geq 30\%$, with one PR and 2 SDs [69].

Aside from the beneficial effects demonstrated in hematological malignancies, MGCD0103 also demonstrated clinical benefits in solid tumor treatment. A phase I trial in patients with advanced solid tumors given MGCD0103 three times per week for 2 of every 3 weeks showed tolerable DLTs of fatigue, nausea, vomiting, anorexia, and dehydration. After four or more cycles, SD was observed in five of 32 patients. A phase II dose of 45 mg/m²/day was recommended [70]. Phase I/II studies in solid tumors were also conducted in combination with gemcitabine. Phase I included patients with refractory solid tumors. Phase II was limited to gemcitabine naive patients with locally advanced or metastatic pancreatic cancer. During a 28-day cycle patients received MGCD0103 three times per week in a dose ascending 3 + 3 design targeting a DLT of <33%. Gemcitabine was administered three times per cycle weekly at 1000 mg/m². Out of the 14 patients evaluated, there were 2 PRs in patients with pancreatic carcinoma, one PR in a patient with nasopharyngeal cancer, and one PR in a patient with cutaneous T-cell lymphoma. The phase II trial is ongoing at a dose of 90 mg for patients with pancreatic cancer [71].

7. Panobinostat (LBH589)

Panobinostat is a hydroxamate that has shown potential in early phase I and II clinical trials. In an initial trial, 15 patients with AML, ALL, or MDS were treated with 4.8 to 14 mg/m² panobinostat administered intravenously as a 30-minute infusion. Transient blast cell reductions occurred in 8 of 11 patients with peripheral blasts. Four patients exhibited a DLT of grade 3 QTcF prolongation at 14 mg/m², which were asymptomatic and cleared after treatment ended. Common toxicities included nausea, diarrhea, vomiting, hypokalemia, loss of appetite, and thrombocytopenia [72]. CTCL patients (stage IB-IVA), including Mycosis Fungoides (MF) and Sezary Syndrome (SS), who have failed two or more previous therapies were enrolled in a phase II clinical trial. Panobinostat was administered at 20 mg orally on days 1, 3, and 5 weekly until disease progression or intolerance to two groups of patients, one who had received prior treatment with oral bexorotene and a second without. The best overall responses were 3 PRs and 4 SDs. ECG monitoring of QTcF prolongation was performed, without any >500 ms [73].

8. Belinostat (PXD101)

Belinostat has shown promising anticancer activity in both hematologic malignancies as well as solid tumors. In a trial enrolling 16 patients with advanced hematological neoplasms, belinostat was administered intravenously at one of three dose levels: 600, 900, and 1000 mg/m²/d. While no CRs or PRs were noted, intravenous administration was

well tolerated, and five patients (including two with DLBCL) achieved SDs after 2–9 treatment cycles. There were no grade 3 or 4 hematological toxicities (except one case of grade 3 lymphopenia), and the most common adverse effects were nausea, vomiting, fatigue and flushing. There were two grade 4 renal failures in patients with multiple myeloma (MM). The recommended dose for phase II studies was 1000 mg/m²/d, intravenously administered on days 1–5 of a 21-day cycle for patients with hematological neoplasia [74].

For solid tumors, Belinostat was tested in a phase I study of patients with advanced refractory cancers. The 46 patients received six dose levels, ranging from 150 to 1200 mg/m²/d over a 5-day cycle. DLTs were fatigue, diarrhea, atrial fibrillation, and grade 2 nausea/vomiting, which led to inability to complete the full cycle. 39% of patients resulted in SD. Of the 24 patients treated at the MTD, which was determined to be 1000 mg/m²/d, 50% achieved SD [75]. Patients with platinum resistant epithelial ovarian cancer (EOC) are resistant to conventional chemotherapy. Belinostat was administered intravenously at 1000 mg/m²/d on days 1–5 of a 21-day cycle to metastatic or recurrent platinum resistant EOC and low malignant potential (LMP) ovarian tumors. Of the 18 patients with LMP, 1 had PR, 10 had SDs. Median PFS in LMP was 13.4 months. Patients with EOC 9 had SD with a median PFS of 2.3 months [76].

9. Entinostat (MS-27-275)

Clinical trials of Entinostat, a benzamide derivative, initiated in 2005 with a Phase I study enrolling patients with advanced solid tumors or lymphoma. Entinostat was administered to a total of 22 patients once a week for 4 weeks during a 6-week cycle. The MTD was determined to be 6 mg/m², and the common DLTs were hypophosphatemia, hyponatremia, and hypoalbuminemia, which were all reversible [77]. After the analysis of three different dose schedules, 4 mg/m² weekly or 2 to 6 mg/m² every other week, for three weeks in a 28-day cycle; the biologically relevant plasma concentrations and antitumor activity were determined [78].

In solid tumors, a phase I combination therapy trial was performed on ten patients with an advanced NSCLC. Patients were treated with 5-azacitidine (AZA), a DNA methyltransferase inhibitor, subcutaneously on days 1–6 and 8–10 along with a fixed dose (7 mg/m²) on day 3 and 10 of a 28-day cycle of entinostat. The dose of AZA was varied by cohort using a standard 3 + 3 dose assessment. No DLTs were observed in the 30 mg/m² dose cohort. However, in the 40 mg/m² cohort, after one week, a patient was replaced due to rapidly progressing disease, and another patient experienced a grade 3 neutropenia and thrombocytopenia. The common toxicities included injection site reactions, nausea/vomiting, constipation, fatigue, and cytopenias. One patient had a PR, which continued longer than 8 months. Two patients had SDs and the remaining patients had PODs [79].

10. Valproic Acid

Valproic acid (VPA) has been increasingly studied in clinical trials for a variety of cancer types as a single agent or in

combination with other therapies. In solid tumors, VPA was analyzed for activity in 12 patients with cervical cancer. Three four-patient dose cohorts were formed, for 20 mg/kg, 30 mg/kg, and 40 mg/kg administered orally for five days over a six-day protocol. Tumor-deacetylase activity decreased in eight patients in a statistically significant manner. A grade 2 depression in level of consciousness was registered in 9 patients [80]. Another phase I study in 26 patients revealed neurocognitive impairment, with grade 3 or 4 neurological side effects in 8 of the 26 patients. When administered intravenously the MTD was determined to be 60 mg/kg/d [81]. A phase II study for the treatment of advanced solid tumors with hydralazine and VPA revealed clinical benefit in 80% (12) of patients with cervix, breast, lung, testis, and ovarian carcinomas. Four patients had PRs and eight SDs, and the most common toxicity was hematological [82].

VPA has been more frequently studied in the use of combination therapies, specifically with all transretinoic acid (ATRA). From a study of 75 patients with AML/MDS, 66 were initially treated with VPA monotherapy followed by ATRA in nonresponsive or relapsed patients. VPA was administered for a median treatment duration of 4 months and ATRA, 2 months. 24% of patients showed hematological improvement with a median response duration of 4 months. Four out of 10 relapsed patients, when administered ATRA had a second response and both treatments were well tolerated [83]. VPA was also combined with both AZA as well as ATRA in patients with AML or high-risk MDS. A total of 53 patients were treated with AZA at the fixed dose of 75 mg/m² daily for 7 days, ATRA at 45 mg/m² orally daily for 5 days starting on day 3, and VPA, which was dose escalated and administered orally daily for 7 days concomitantly. The ORR was found to be 42%, the median remission duration was 26 weeks, the MTD for VPA was 50 mg/kg daily for 7 days and the DLT was reversible neurotoxicity [84]. In another study of patients with AML/MDS, increasing doses of VPA administered orally and concomitantly with a fixed dose of decitabine (15 mg/m² by intravenous daily infusion) for 10 days revealed a safe daily dose of 50 mg/kg. 22% (12) of patients had an objective response, this included 10 CRs and 2 CRs with incomplete platelet recovery [85].

11. Associations of HDACs Inhibitors with Other Target Drugs

Despite the very high number of gene products potentially deregulated in solid tumors, high throughput screening analyses suggest that mutations often occur in genes that collaborate in a relatively limited pool of common cell signaling pathways [86]. This hypothesis may have a great relevance in the clinic. In fact, having at hand several classes of effective “pathway-oriented” target drugs, and admitting that a tumor may be driven by a limited number of deregulated pathways, it possible that the concomitant use of a combination of drugs directed against different pathways functionally related may result in an improved antineoplastic effect or in the overcoming of drug resistance.

Recent studies on multiple myeloma (MM) models suggest that HDACs inhibitors may synergize with proteasome

inhibitors. Although the molecular mechanism underlying this effect is not completely understood several means have been proposed [87] and encouraging data has come from the early clinical experimentation, including a phase I trial [88] of randomized patients with relapsed/refractory MM to receive Vorinostat (200 mg twice daily or 400 mg once daily for 14 days) in combination with bortezomib (0.7 or 0.9 mg/m² on days 4, 8, 11, and 15 or 0.9 or 1.1 or 1.3 mg/m² on days 1, 4, 8, and 11). Among 34 evaluable patients, the best response to vorinostat plus bortezomib was a partial response (PR) in 9 (26%) patients, minimal response (MR) in 7 (21%) patients, and stable disease (SD) in 18 (53%) patients. Mean duration of SD was 89 days, range 9–369 days. Of the 13 evaluable patients who had previously been treated with bortezomib, 5 achieved a PR, 1 had an MR, and 7 had SD. Eleven of the 34 patients enrolled (32.4%) discontinued treatment due to adverse effects (AEs). Most common AEs were fatigue, nausea, diarrhea, and hematological toxicities. A phase II open label study from the same group is currently ongoing. Another Phase I trial accrued 23 heavily pretreated (median of 7 previous regimens) patients with relapsed/refractory MM to receiving escalating doses of Bortezomib (1 or 1.3 mg/m² on days 1, 4, 8, and 11 and Vorinostat 100 mg twice daily, 200 mg twice daily, and 400 mg once daily, or 500 mg once daily for 8 days each 21-day cycle). Overall response rate was 42%, two patients receiving 500 mg vorinostat had prolonged QT interval and fatigue as dose-limiting toxicities. The most common grade >3 toxicities were myelosuppression ($n = 13$), fatigue ($n = 11$), and diarrhea ($n = 5$). In the same setting of patients with relapsed/refractory MM, the combination of Romidepsin and Bortezomib and Dexamethasone has also shown promising results. In a Phase I/II trial, of 18 evaluable patients, this schedule resulted in an overall response rate of 67%. The most common drug related grade 3 toxicities included fatigue (2 pts.), neutropenia (1 pts.), sepsis (2 pts.), and peripheral neuropathy (1 pts.). Preclinical data seems to confirm a synergic effect of Panobinostat and Bortezomib, and a Phase I trial is currently ongoing (NCT00532389). These encouraging results are paving the way to a relevant number of trials testing the association of different HDAC and Proteasome inhibitors, and results are expected in a relatively short time.

12. HDACs Inhibitor-Related Toxicity

The relationship between the toxicity of HDACs inhibitors and their pharmacodynamic/pharmacokinetic properties is still largely unknown. This makes it difficult to optimize HDACs inhibitors treatment. Studies in preclinical models have shown that HDACs inhibitors are a class of agents that has been generally well tolerated and proved a very good toxicity profile in comparison with other chemotherapeutic drugs used in cancer therapy. The main adverse effect is fatigue, which is generally mild and tolerable in most patients, but in 30% of patients, it can be severe enough to cause drug discontinuation. Gastrointestinal toxicities are also common side effects and include anorexia, nausea, vomiting, and diarrhea. Overall, they are mild and controllable

with symptomatic treatment. Biochemical disorders such as hypokalemia, hyponatremia, hypocalcemia, hyperglycemia, hypophosphatemia, and hypoalbuminemia are common with various HDACs inhibitors, while neurocortical disturbances including somnolence, confusion, and tremor are observed mainly with phenylbutyrate and valproic acid. All these side effects are generally reversible upon cessation of administration of the drug.

Another side effect of histone deacetylase inhibitors is transient thrombocytopenia that is relatively common with most HDACs inhibitors [89], it is generally mild, although has been dose limiting in some studies.

A significant adverse reaction regards the cardiotoxicity. Early studies in preclinical animal models have shown that various HDACs inhibitors such as Romidepsin are able to cause myocardial inflammation and cardiac enzyme elevation. These studies represent a controversial issue since high doses of HDACs inhibitors were used [90, 91] compared to the doses that were confirmed appropriate for use in Phase I trials. Specifically, the effect of Romidepsin on cardiac function was assessed in 42 patients with T-cell lymphoma. They received a total of 736 doses of Romidepsin and an intensive cardiac monitoring was evaluated [92]. Grade I (T-wave flattening) and grade II (ST segment depression) ECG changes occurred in more than half of the ECGs obtained post treatment; however, these changes were reversible and of short duration, with no elevation in cardiac enzymes and no significant changes in left ventricular ejection fraction.

In addition, cardiac dysrhythmias were observed in a small number of patients but most of these patients had pretreatment documented dysrhythmias. Similar ECG changes and QT-interval prolongation have been reported in other Phase I/II Romidepsin studies [60, 66, 93–96]. In other Romidepsin studies, there have been reports of sudden death; however, the relationship to the drug remains unclear. In particular, a Phase II study of 15 patients with metastatic neuroendocrine tumors, administered with standard doses of Romidepsin reported one sudden death in a 48-year-old patient [66]. However, this patient had a history of hypertension, and a biventricular hypertrophy was revealed by postmortem examination, both are known risk factors for sudden death. Cardiotoxicity may be a class effect of HDACs inhibitors, being more frequent with Romidepsin and other class-I inhibitors rather than Vorinostat and other pan-HDAC inhibitors but it is unlikely that these side reactions are limited just to those HDACs inhibitors. Additional parallel cardiotoxicity studies with other various HDACs inhibitors are necessary.

Possible room for improvement could be in the development of isoform-selective HDACs inhibitors (extensively reviewed in [97]). It is known from knockout studies that the deletion of some specific HDACs isoforms can cause precise phenotypic defects. In particular, mice lacking some of the HDACs isoforms (namely, HDAC2, HDAC3, HDAC5, and HDAC9) show severe cardiac malformations and dysfunctions [98, 99], suggesting that HDACs inhibitors, specific for other HDACs could possibly have a better cardiotoxicity profile still retaining the full pro-apoptotic action. Furthermore the introduction of reliable sensitivity

biomarkers in the design of trials will allow a better stratification of patients thus minimizing the risk of exposure of the unresponsive subjects to HDACs treatment and toxicity. Recently, a genome-wide loss-of-function screening was undertaken to reveal genes that govern tumor cell sensitivity to HDAC inhibitors in a sarcoma cell model, and HR23B, a protein involved in shuttling ubiquitinated proteins to the proteasome was identified as a potential biomarker [100]. HR23B expression was further investigated in 21 skin biopsies from 20 patients with CTCL enrolled in a Vorinostat Phase II trial [46] and analyzed by immunohistochemistry. The proportion of patients with a strong HR23B staining who had a clinical response was 69%, thus suggesting a pretty high positive predictive value (PPV). Similar PPV for HR23B were obtained when looking at patients treated with other HDACs inhibitors [101].

13. Conclusions

HDAC, inhibitors represent a promising new group of anticancer agents, even though the mechanisms of HDAC inhibitor-induced tumor cell death require further elucidation. While vorinostat and romidepsin are the only US FDA-approved HDACs inhibitors currently utilized in cancer therapy, as we have shown here, there are many HDACs inhibitors that are presently under intense clinical investigation, both as single agents and combination therapies. These will hopefully be able to further improve the range of treatment options available for hematologic malignancies as well as for solid tumors.

As we come closer to understanding the molecular mechanisms inherently responsible for tumorigenesis, as well as the full range of HDACs inhibitor cellular actions, we will be able to target in a more appropriate way and be able to pair cancer therapies for clinical use. In order to establish rigorous patient selection criteria and optimal drug combinations to properly design further trials and maximize the clinical gain, the bridge between the biological function and the therapeutic benefit of these drugs needs to be further elucidated.

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Review Article

Histone Deacetylase Inhibitors: The Epigenetic Therapeutics That Repress Hypoxia-Inducible Factors

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Histone deacetylase inhibitors (HDACIs) have been actively explored as a new generation of chemotherapeutics for cancers, generally known as epigenetic therapeutics. Recent findings indicate that several types of HDACIs repress angiogenesis, a process essential for tumor metabolism and progression. Accumulating evidence supports that this repression is mediated by disrupting the function of hypoxia-inducible factors (HIF-1, HIF-2, and collectively, HIF), which are the master regulators of angiogenesis and cellular adaptation to hypoxia. Since HIF also regulate glucose metabolism, cell survival, microenvironment remodeling, and other alterations commonly required for tumor progression, they are considered as novel targets for cancer chemotherapy. Though the precise biochemical mechanism underlying the HDACI-triggered repression of HIF function remains unclear, potential cellular factors that may link the inhibition of deacetylase activity to the repression of HIF function have been proposed. Here we review published data that inhibitors of type I/II HDACs repress HIF function by either reducing functional HIF-1 α levels, or repressing HIF- α transactivation activity. In addition, underlying mechanisms and potential proteins involved in the repression will be discussed. A thorough understanding of HDACI-induced repression of HIF function may facilitate the development of future therapies to either repress or promote angiogenesis for cancer or chronic ischemic disorders, respectively.

1. Introduction

Tumors are one of the leading causes of disability and mortality in the USA and other developed countries. While many advances have been made in both basic research and clinical treatment, the development of more efficient cancer-specific therapies remains an unfinished mission. In addition to surgery and radiation therapy, chemotherapy is an important component in treating a variety of cancers, particularly for late stage, advanced cancers that are unsuitable for surgical removal. Chemotherapeutics are commonly antiproliferative compounds that preferentially kill dividing cells, rarely discriminating cancer cells, or normal dividing cells such as hematopoietic cells. Given sufficient dose and time, chemotherapeutics should be able to kill all cancer cells theoretically. However, in clinical practice, two of the major hurdles of chemotherapy are (1) tumor hypoxia,

which is related to inefficient drug delivery and triggers drug resistance [1] and (2) adverse effects on normal tissues, which frequently limit the dose and duration of treatment. These two hurdles limit the efficacy of chemotherapy. To overcome these hurdles, an emerging trend in cancer therapy is to specifically target hypoxic cancer cells [1, 2]. Indeed, hypoxia, HIF activation, and angiogenesis in solid tumors have been demonstrated by many independent studies [3–5]. Particularly, hypoxic and angiogenic tumors are usually resistant to traditional radiation and chemotherapy [6–10]. Blocking tumor angiogenesis has been extensively explored as a novel treatment for cancers in the past decade. The identification of HIF-function as the master regulator of angiogenesis and tumor cells adaptation to various stress conditions, including those caused by chemotherapy and radiation, provides the rationale to target HIF function as an important part in cancer therapy. Since HIF function is

TABLE 1: Histone deacetylases: Classification and characteristics.

Classes	HDACs	Localization	Features
Class I	Ia	HDAC 1, 2	Nucleus
	Ib	HDAC 3	Nucleus & cytoplasm
	Ic	HDAC 8	Nucleus
Class II	IIa	HDAC 4, 5, 7, 9	Nucleus/cytoplasm [58]
	IIb	HDAC6	Cytoplasm
		HDAC10	Nucleus/cytoplasm [59, 60]
Class III	Sirtuins (Sirt1-7)	Nucleus, cytoplasm & mitochondria	Related to the Sir2 NAD ⁺ -dependent [25]
Class IV	HDAC 11	Nucleus & cytoplasm	Features of both classes I and II [61], Zinc independent

essential for both tumor progression and tissues' adaptation to chronic ischemia, it is a potential therapeutic target not only for cancer but also for chronic ischemic disorders.

In recent years, several HIF inhibitors have been identified by compound screening processes [11–13]. Interestingly and surprisingly, basic research and clinical trials have shown that HDACs block angiogenesis and suppress tumor growth [14–16]. It has been gradually realized that these effects are at least partially mediated by repressing HIF function. Specifically, a unique phenomenon has been reported that inhibitors of class I/II HDACs, which usually stimulate transcription factors, repress the transactivation potential of both HIF-1 α and HIF-2 α [17]. Importantly, HDACs repress HIF- α in all cells examined, indicating a ubiquitous mechanism [17, 18]. Although HDACs were originally designed as epigenetic therapeutics, the effects of these compounds are generally pleiotropic. The direct molecular targets of HDACs and the biochemical mechanisms underlying the repression of HIF function remain elusive. In this paper, we will first briefly summarize HDACs, HDACs, and the regulatory mechanisms of HIF function. We then will focus on analyzing the potential links between protein hyperacetylation triggered by inhibitors of type I/II HDACs and its repressive effect on HIF function.

2. Histone Deacetylases and Histone Deacetylase Inhibitors

HDACs compass a large family of enzymes that remove the acetyl groups from N- ϵ -lysines of histones [19–21]. Since the original discovery of histone acetylation, nonhistone proteins such as transcription factors or coactivators have been shown to be subjective to the same modification. Therefore, HDACs are now redefined as lysine deacetylases to more precisely reflect the fact that its substrates, acetylated lysyl residues, are not exclusive for histones [22]. Acetylation status of these proteins is usually reversibly regulated by a dynamic balance between acetyl transferases (HATs) and HDACs.

So far 18 HDACs have been identified from mammalian cells, which are classified into four classes based on their homology to yeast enzymes [23–25] (Table 1). HDAC1-3, 8 are nuclear localized class I HDACs and are most commonly associated with transcription repressive complexes known as Sin3, NuRD, CoRest (HDAC1, 2), and SMRT/NCOR

(HDAC3) [19–21, 26, 27]. Generally, Class I HDACs are considered to be repressive factors for gene expression, despite a few exceptions [28, 29]. HDAC1 is also known to inhibit the function of the phosphatase PTEN involved in cell signaling by deacetylation [30]. HDAC3 is reported to control the acetylation of p65, the subunit of NF- κ B, which is a key transcription factor involved in responses to inflammation and other cellular stresses [31]. HDAC4–7, 9, 10 belong to class II [20]. HDAC4 is involved in a multiprotein transcriptional corepressor complex and is implicated in myocyte differentiation, skeletogenesis, and neuronal survival [32–35]. HDAC5 has been suggested to interact with nuclear receptor corepressors 1 and 2, which are important in the down regulation of gene expression [36]. A key role in development and pathophysiology of cardiomyocytes has been proposed for HDAC5 [37, 38]. As an exclusive member mainly functioning in the cytoplasm, HDAC6 deacetylates cytoplasmic nonhistone substrates including Hsp90 [23, 39] and α -tubulin [40–43]. HDAC6 also binds to misfolded proteins and dynein motors, thus allowing the misfolded proteins to be physically transported to molecular chaperones and proteasomes for degradation [44]. Class III comprises of the NAD⁺-dependent Sirt1-7 [45–49], which has been implicated in caloric restriction, aging, neuronal degeneration, and longevity [50–52]. HDAC11, which is sometimes called class IV [53], negatively regulate interleukin 10.

HDAC inhibitors (HDACI) encompass several diverse compounds that inhibit deacetylases. Several HDACIs commonly seen in literatures are listed in Table 2, and the chemical structures of representative compounds are shown in Figure 1. Since protein acetylation *in vivo* is, in most cases, reversibly regulated by a dynamic balance between histone acetyl transferases (HATs) and HDACs [19–21], exposure of cells to HDACIs breaks the balance and induces hyperacetylation of proteins. Similar to enhanced HAT activity, HDACs generally promote gene expression by elevating the acetylation status of histones, transcription factors, and coactivators. Importantly, HDACIs are anticancer compounds undergoing intensive investigation; some of them have been approved by the US Food and Drug Administration (FDA) for clinical treatment of certain types of cancer patients. Clinical and experimental data show that inhibitors of class I/II HDACs repress tumor growth and induce apoptosis.

TABLE 2: Major HDAC inhibitors: Targets and current status in cancer chemotherapy.

Class	Compounds	HDACs	Status	Reference
Aliphatic acid	Valproic acid	Class I, IIa	Phase II clinical trials	[62]
Benzamide	MGCD0103	Class I, II	Phase II clinical trials	[63]
Cyclic peptide	FK228	Class I, II	FDA approved for CTCL	[64]
	SAHA	Class I, II	FDA approved for CTCL	[63]
Hydroxamates	LBH589	Class I, II	Phase II and III clinical trials	[23]
	Trichostatin A	Class I, II	Experimental use	[65, 66]
Others	AR-42	Class I, II	Started clinical trials	[67]
	CUDC101	Class I, II	Started clinical trials	[68]

Note: Valproic acid has been in use as an anticonvulsant and mood stabilizing drug in the treatment of epilepsy and bipolar disorder.

While mainly considered as epigenetic therapeutics, HDACs enhance the level of acetylation of nonhistone proteins as well. For example, the acetylation states of the transcription regulators such as *c-Myc*, E2F1, HNF-4, Ku70, NF- κ B, p53, RB, Runx, Sp3, STATs, and YY1 are affected by HDACs [14, 54]. It is important to note that HDACs may also affect the acetylation of cytoplasmic/mitochondrial proteins that are not directly involved in the transcriptional control of gene expression [55, 56].

3. Hypoxia, Hypoxia Inducible Factors, the Oxygen Sensing Pathway, and Angiogenesis

The oxygen-sensing pathways, which represent the canonical regulatory mechanism of HIF function, have been investigated in depth, making it possible to modulate HIF function as a novel therapy. Hypoxia-inducible factors (HIF-1, 2, 3) are heterodimeric transcription factors, each composed of a unique α -subunit (HIF-1 α , 2 α or 3 α) and a common β -subunit (HIF- β) shared by HIF- α and other transcription factors. HIF-1 and HIF-2 are the major contributors to the transcription of HIF target genes that encompass several orchestrated functional groups [69, 70]. While regulating the expression of overlapping target genes, HIF-1 and HIF-2 have been demonstrated to possess distinctive nonredundant functions [71–73]. The overall biological effect of expression of HIF target genes is to facilitate the utilization of oxygen and other nutrients, thus inducing cellular adaptation to hypoxia, chemotherapy, and other cellular stresses [69, 70]. Most importantly, the expression of key proangiogenic factors, such as vascular endothelial growth factor (VEGF) [74, 75], bFGF, and their receptors [76, 77], stimulates angiogenesis and vasculogenesis, which are fundamental processes involved in tumorigenesis, wound healing, chronic ischemic adaptation, and early embryonic development.

As heterodimeric transcription factors, HIF-1 and HIF-2 are functionally controlled by their alpha subunits (HIF-1 α and HIF-2 α , resp., HIF- α collectively). HIF- α activity is controlled by two well-known mechanisms [78–80] (Figure 2). Firstly, HIF- α is rapidly degraded through a hydroxylation-ubiquitination-proteasomal system (HUPS) when oxygen is sufficient. With an adequate oxygen supply, HIF- α is hydroxylated at two prolyl residues in the oxygen-dependent degradation domain (ODD) by a family of prolyl hydroxylases

(PHD) [78–80]. The oxygen-facilitated hydroxylation makes HIF- α recognizable by VHL, an E3 ligase, for ubiquitination [78–80]. Consequently, the ubiquitinated HIF- α is degraded by the proteasome system [81, 82]. Secondly, HIF- α activity is controlled by its transactivation potential (TAP), which is in turn controlled by its interaction with coactivator p300/CBP and other factors [83, 84]. Factor inhibiting HIF-1 (FIH), an oxygen-dependent hydroxylase, modifies an Asn residue at the carboxyl terminal activation domain (HIF- α CAD) and disrupts its interaction with p300/CBP [85, 86]. In addition, HIF- α has an N-terminal activation domain (NAD) whose activity is also influenced by oxygen availability. Lack of oxygen (hypoxia, anoxia), a common pathophysiological condition frequently complicated with neoplastic, cardiovascular, hematologic, and respiratory disorders, represses the activity of hydroxylases and activates HIF function [78–80]. Finally, hypoxia triggered generation of reactive oxygen species by the mitochondrial electron transfer chain has been identified as a major player in the stabilization of HIF- α [87, 88]. Oxygen and oxygen-dependent hydroxylation-triggered events form the conventional regulatory pathways of HIF function (Figure 2), illustrating a physiological feedback.

HIF activation and the expression of HIF target genes play key roles in tumorigenesis and angiogenesis. One of the major metabolic features of tumors is that they usually demand increased oxygen, carbon, and nitrogen sources because of active biosynthesis during cell growth and proliferation [89]. Continuous growth of primary or metastatic tumors can happen only when this demand is met, usually by angiogenesis. Indeed, hypoxia and HIF-1 activation has been observed in a variety of solid human tumors [90–93], accompanied by overexpression of HIF target genes and angiogenesis. Loss of HIF-1 α dramatically retards solid tumor growth *in vivo* and is correlated with a reduced capacity to release proangiogenic factors [94–96]. Angiogenesis is defined as the formation of new blood vessels from pre-existing vessels. Neoplastic angiogenesis involves three major components [97]: (1) the tumor cells that synthesize and secrete signaling molecules and growth factors (paracrine), (2) the extracellular matrix and surrounding microenvironment, and (3) the responses of endothelial and other stromal cells. It is noted that tumor-secreted signaling molecules not only function on endothelial cells,

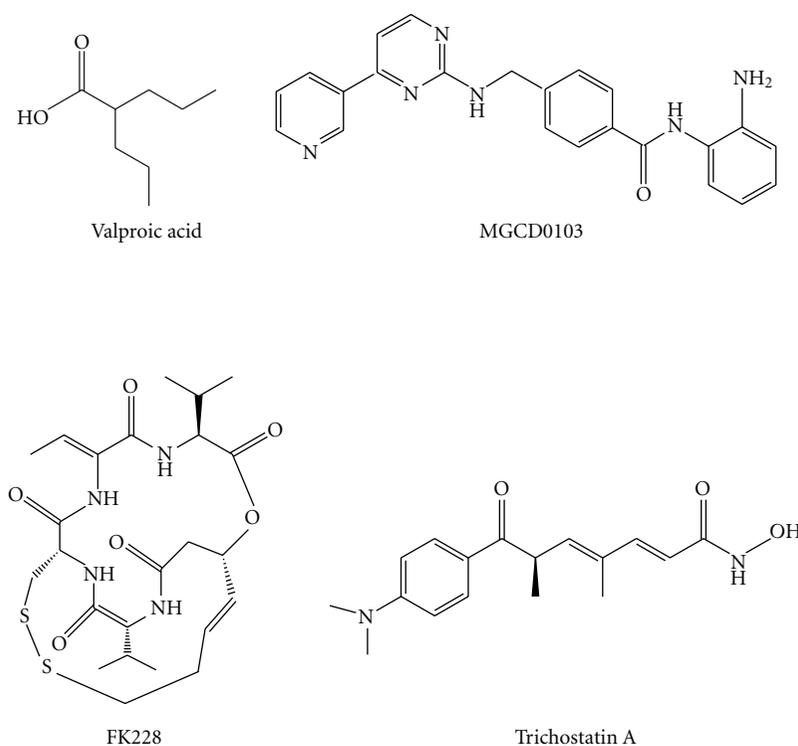


FIGURE 1: Chemical Structures of Representative Inhibitors of Class I/II HDACs. While structurally diverse, they share one common feature: the existence of active groups targeting the zinc-dependent catalytic sites of class I/II HDACs.

but also satisfy the growth factor requirement of tumor cells. The HIF-stimulated autocrine loop renders tumor cells independent of growth factors from other origins. Therefore, HIF-induced angiogenesis and secretion of growth factors fulfill the major needs of sustainable tumor growth, invasion, and progression. While expression of HIF-1 α and HIF-2 α has been observed in both stromal and tumor cells [71–73, 98, 99], an intrinsic or autonomous role of HIF-2 α in endothelial cells has been also reported [72]. HIF-1 α is believed to play more important roles in regulating tumor cell survival and metabolic reprogramming in response to hypoxia [94–96].

Hypoxia is not the only cause of HIF activation in tumors. In solid tumors, in addition to hypoxia, a combination of extracellular and intracellular factors (Figure 3), including growth factors, mitogenic signaling (MAPK, PI3K/Akt), activation of oncogenes, and loss of tumor suppressors (VHL, p53 and PTEN), activates HIF- α by acting on various points of the canonical pathways [78–80, 100–106]. Considering the complexity of signaling pathways that lead to HIF-activation in tumors, it is generally difficult to repress HIF function by repairing the aberrant canonical pathways. Furthermore, the diverse contributions of HIF target genes to metabolic reprogramming, cell survival, tumor growth, and progression make it less effective to block each effect of HIF activation. Instead, directly targeting HIF may be an ideal strategy for cancer therapy. Currently, many studies are actively exploring compounds to directly repress HIF- α , and several HIF inhibitors have been developed [11–13].

Interestingly, some small molecular weight compounds under development for cancer therapy but not originally intended to target HIF function show good anticancer effects and antiangiogenic features. These include HDACIs [14–16], heat shock protein (HSP) 90 inhibitors [107, 108], proteasome inhibitors [109–112] and microtubule inhibitors [113–115]. While these compounds are aimed at distinct cellular targets, studies have linked their antiangiogenic and antitumor effects to HIF inhibition.

4. Histone Deacetylase Inhibitors Repress Tumor Angiogenesis and HIF Function

Accumulating evidence suggests that inhibition of class I/II HDAC activity represses HIF function in tumor cells [17, 18, 116–122]. The HDACIs showing anti-HIF activity generally block class I and II HDACs. While most inhibitors of class I/II HDACs are not selective for a particular deacetylase, they do not directly repress the enzymatic activity of class III HDACs (Sir2 family) [48, 49, 123]. Trichostatin A (TSA) is among several HDACIs reported to repress angiogenesis *in vitro* and *in vivo* [118, 124]. Other HDACIs including FK228 (depsipeptide, FR901228) [120, 121], butyrate [28, 122], and LAQ82481 have been known to repress angiogenesis and expression of HIF regulated pro-angiogenic factors, such as vascular endothelial growth factor (VEGF). While HIF1 had been accepted as a major regulator of angiogenesis, the early explanations for the antiangiogenesis effects of HDACIs

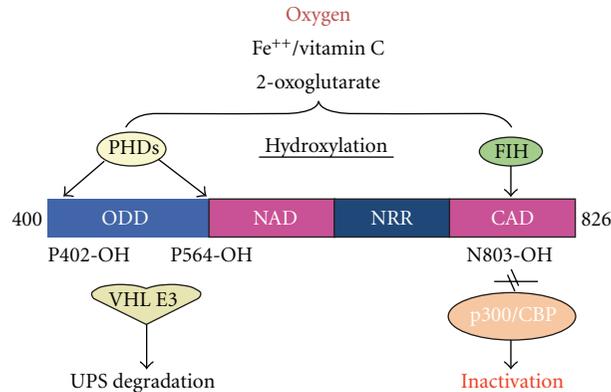


FIGURE 2: Regulation of HIF-1 α by Oxygen-Dependent Hydroxylation. HIF function is continuously regulated by the concentration of molecular oxygen, representing an essential part of physiological feedback loop. In this feedback loop, oxygen sensing is achieved by oxygen-dependent hydroxylation of specific amino acid residuals of HIF- α . Hydroxylation of two prolyl residuals leads to ubiquitination and proteasome-dependent degradation of HIF- α . Hydroxylation of an asparagine residual located at the CAD by FIH impairs its interaction with coactivator p300 or CBP, thus repressing the transactivation activity. Note that the hydroxylation reactions require ferrous ion and ascorbic acid as cofactors, and 2-oxoglutarate as cosubstrate.

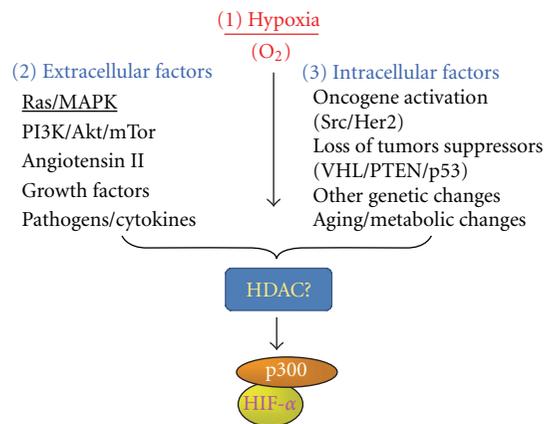


FIGURE 3: Multiple Signaling Pathways Regulate HIF Function and Key Determinants of the Transcription Activity of HIF-1 and HIF-2. The overall function of HIF complexes is mainly determined by protein levels of the α -subunits (HIF- α) and their interactions with p300 or CBP. Multiple signaling pathways may modulate HIF function either through acting on HIF- α (stability or simple posttranslational modifications) or on p300/CBP (posttranslational modifications). Eventually, the HIF- α -p300 or HIF- α -CBP complexes serve as the integrators of these signals and coordinate the dynamic reprogramming of gene expression. HDACs may directly interact with the HIF transcription complexes, or indirectly or functionally interact with these signaling pathways and regulate HIF function.

varied, perhaps because HDACs have a pleiotropic nature, and multiple pathways regulate angiogenesis. Later findings suggest that HDACI-mediated repression of angiogenesis renders its effect on HIF function in tumor cells [118, 120, 124–127]. While detailed molecular and biochemical mechanisms remains unknown, several current explanations include (1) HDACI-mediated destabilization of HIF-1 α [18–21, 26–29, 40–43, 48, 49, 53, 116, 118, 123, 124, 128], (2) HDACI-mediated repression of the transactivation potential of the carboxyl-terminal transactivation domain of HIF- α CAD [17, 129], (3) repressing DNA binding ability [120], and (4) inhibiting nuclear translocation of HIF-1 [117, 122]. Below we will focus our discussion on HDACI-mediated destabilization of HIF-1 α and HDACI-mediated

repression of HIF- α CAD TAP, the two better supported models. We will discuss data consistent with or contrary to these views. Interested readers are referred to other proposed mechanisms including inhibiting nuclear translocation of HIF-1 α [117, 120, 122].

5. Mechanisms Underlying HDACI-Mediated Repression of HIF- α Transactivation Potential

Early report suggested that TSA repressed angiogenesis by regulating VHL and p53 function, hence destabilizing HIF-1 α [118]. Later observations show that HDACs also repress

the TAP of the carboxyl-transactivation domain (CAD) of both HIF-1 α and HIF-2 α [17]. This effect can be clearly demonstrated by using a recombinant HIF- α CAD construct fused to the DNA binding domain of the yeast GAL4 transcription factor. The protein levels of this fusion protein are not decreased by HDACs, allowing the examination of its activity by monitoring the expression of a reporter gene [17]. All other transactivators tested in the same way, including p300, VP16, MyoD, and p53, were enhanced by HDACs under the same conditions. The effects of HDACI on the transactivation potential have two special features that are distinct from the destabilizing effects. First, low doses of HDACs that were not sufficient to cause HIF-1 α degradation were sufficient to repress HIF-1 α transactivation potential under both normoxic and hypoxic conditions [17]. Second, while HDACs repress the transactivation potential of both HIF-1 α and HIF-2 α , they only trigger the destabilization of HIF-1 α , not HIF-2 α [17, 18]. Because of these two features, this mechanism may be more relevant to the antitumor effects of HDACs than the HIF-1 α destabilization caused by high doses of HDACs, because it is easier and more practical to achieve a low therapeutic dose in a clinical setting. Scientifically, this is also interesting because it shows the uniqueness of HIF- α among other transcription factors.

It has been well established that HIF function is determined by the protein levels and the transactivation activity of HIF- α . HIF- α has two transactivation domains, the NAD and the CAD. The transactivation activity of CAD is absolutely dependent on the interaction of the CAD with either p300 or CBP. The interaction between HIF-1 α and p300 (or CBP) requires an intact CH1 domain of p300 (or CBP). In addition, HIF-1 α has been reported to possess a p300/CBP CH1-independent transactivation activity which is also sensitive to HDACs [129, 130]. Because HIF- α CAD has been demonstrated to be absolutely dependent on p300/CBP CH1 [129], the p300/CBP CH1-independent mechanism might involve the NAD of HIF- α . These reports indirectly indicate that inhibitors of class I/II HDACs also repress the transactivation activity of HIF- α NAD.

Because HDACs mediate repression of HIF function in a manner independent of HIF- α levels, the key targets of this repression must be the HIF- α -p300 or HIF- α -CBP complexes (Figure 3). In oxygen-sensing pathway, oxygen availability regulates this interaction through FIH (Factor inhibiting HIF-1)-mediated hydroxylation of HIF- α CAD. However, mutation of Asn803 of HIF-1 α CAD did not abolish HDACI-mediated repression [17], indicating that the HDACI-mediated repression of HIF-1 α -p300 function is independent of either FIH or hydroxylation. The HDACI-mediated repression of HIF- α TAP is also independent of VHL [17], suggesting a mechanism distinct from the normoxic repressive pathway. Since a minimal CAD domain (HIF-1 α 786-826) lacking the normoxic repressive region thus being constitutively active can be repressed by HDACs [17], it is unlikely that the HDACI-mediated repression of HIF- α CAD involves a direct change of acetylation states of HIF- α [17]. HIF- α NAD, on the other hand, overlaps with the oxygen-dependent degradation domain and contains more than one lysyl residues. So it is possible that acetylation

of any of the lysyl residues affects NAD transactivation activity.

While direct acetylation of HIF- α , if any, is unlikely to be involved in HDACI-mediated repression of HIF function, the direct acetylation of p300/CBP, the other determinant of the transactivation activity of HIF complexes, has been well documented. p300 and CBP are acetyltransferases serving as general cofactors for multiple transcription factors including HIF- α [131]. These two proteins possess multiple domains that function as docking sites for their interaction with a variety of transcription regulators [131]. Interestingly, all those important functional domains are lysine-rich and have shown to be subjective to autoacetylation by p300 or CBP [131, 132]. Importantly, exposure of cells to HDACs causes hyperacetylation of p300 [131]. Consistent with these observations, p300 has been reported to complex with HDAC activities [133–135]. These observations suggest that HDACI-mediated repression of HIF transactivation more likely implicates the acetylation status of p300 or CBP. A recent work revealed that the transactivation activity of HIF- α NAD also requires an interaction with p300 or CBP [136]. This interaction is mediated by CH3 domain, which is also one of the lysine-rich regions subjective to acetylation [131]. Therefore, it is possible that the HDACI-mediated repression of HIF- α NAD also involves the acetylation status of p300 or CBP. Considering that both CH1 and CH3 domains of p300 or CBP are lysine-rich and subjective to acetylation [131] and p300 or CBP physically interacts with deacetylase activity [134], one intriguing hypothesis would be that the acetylation status of CH1 and CH3 may affect their binding affinity to different transcription factors [137]. If it is true, acetylation of p300 and CBP may represent an additional mechanism for these two general coactivators to dynamically coordinate the transcriptional reprogramming of multiple genes. Finally, since multiple signaling pathways regulate HIF- α -p300 complex, it is also possible that one or more signaling pathways are relayed by HDAC activity, or some regulators of the signaling pathways are subjective to acetylation (Figure 3).

6. Mechanisms Underlying HDACI-Mediated Degradation of HIF-1 α

As histone acetylation is generally associated with enhanced gene transcription, it is common to find that HDACI enhances the transcription and *de novo* synthesis of proteins. It is also true in most exogenous gene expression systems including transfection of cultured cells and *in vivo* gene therapy. The transcription of endogenous HIF-1 α , however, is not affected by HDACs (Chen & Sang, unpublished data). Previous studies from our laboratories and others have shown that HDACI treatment has little effect on the *de novo* translation of endogenous HIF-1 α protein [137]. Here we focus our discussion on HDACI-mediated degradation of HIF-1 α .

6.1. Do Inhibitors of Class I/II HDACs Directly Enhance the Acetylation of HIF-1 α at Lys532? Interaction between protein acetylation and ubiquitination has been discussed in two

recent reviews [138, 139]. In an early report from Dr. Kim's group, the shorter mouse variant isoform mARD1225, which is a mammalian orthologue of a yeast N- α -acetylase, catalyzed N- ϵ -acetylation of HIF-1- α ODD at Lys532, promotes HIF-1 α recognition and ubiquitination by VHL [124]. The longer human hARD1₂₃₅ isoform is also known to associate with HIF-1 α ODD *in vitro* and with full length HIF-1 α *in vivo* [140]. Subsequent evidence has shown that hARD1 cannot acetylate human HIF-1 α *in vitro* [140–143]. One explanation for this discrepancy is that mARD1₂₂₅ has a C-terminal region that significantly differs from those of other mouse or human ARD1 [144]. An alternative possibility is that hARD1 may aggregate *in vitro*, and aggregated hARD1 loses its catalytic activity as an α -acetylase [145]. Silencing of hARD1 with siRNA affected cell proliferation, but showed no effect on HIF-1 α stability [141, 142]. The role of hARD1 in cell proliferation was further demonstrated in mouse xenograft tumor model [146]. Therefore, while published data suggest that mARD1₂₂₅ has a role in HIF-1 α stability, and hARD1 is implicated in the regulation of cell proliferation, a precise role of hARD1 in HIF-1 α stability remains unclear.

HIF-1 α is easily detectable from the immunoprecipitates by using anti-acetyl-lysine antibodies [116, 124, 147, 148]. It is also possible that HIF-1 α interacts with one or more acetylated proteins, thus is indirectly coprecipitated by antily-sine antibody in immunoprecipitation experiments. More recently, several studies showed direct detection of HIF-1 α in immunoblotting with anti-acetyl-lysine antibodies [149]. The involvement of Sirt1, a member of class III deacetylase, in the regulation of HIF-2 α has been reported [150]. These reports generally support that HIF-1 α may undergo direct acetylation in cells. Nevertheless, a specific role of Lys532 in HDACI-triggered HIF-1 α degradation remains unclear. ODD is sufficient to mediate the HDACI-triggered HIF-1 α instability; however, mutation of the putative acetylation site (Lys532 to Arg) failed to protect ODD from HDACI-induced degradation [18]. So far there is no direct evidence to support that HDACIs of class I/II enhance HIF-1 α acetylation at Lys532 in cells. Mass-Spectrometry analysis of HIF-1 α isolated from cells may eventually resolve the acetylation status of Lys532 of HIF-1 α and shed light on its role in HIF-1 α stability.

6.2. HDACIs Induce Ubiquitination-Independent Degradation of HIF-1 α . In the original model proposed by Dr. Kim, HIF-1 α acetylation at Lys532, either catalyzed by mARD1 or induced by HDACIs, promoted HIF-1 α recognition and eventual ubiquitination by VHL [118, 124]. Since HDACIs enhance the interaction between HIF-1 α and HSP70, an alternative ubiquitination pathway mediated by HSP70-associated CHIP has been proposed [151]. Ubiquitination of proteins sequentially involves three enzyme activities termed E1, E2, and E3. Mammalian cells have a single ubiquitin-activating enzyme E1, and VHL is the HIF- α -specific E3 ligase. Accordingly, VHL-defective cells or E1-inactivated cells accumulate high levels of HIF- α . If HDACI-triggered HIF-1 α degradation was mediated by the canonical ubiquitination pathway, the process would depend on functional

E1 and VHL activity. In fact, it is reported that HDACIs decreased HIF-1 α levels in all cells tested, including VHL (-/-) C2 and RCC4 cells, indicating that HDACI-induced HIF-1 α degradation is through a mechanism existing in, perhaps, all tumors, including those lacking VHL. A special cell line, Ts20TGR, contains a temperature sensitive E1 [152]. Inactivating E1 in this cell line by culturing the cells at 39°C resulted in accumulation of nonubiquitinated HIF-1 α [18]. Apparently, HDACIs are able to trigger degradation of the accumulated nonubiquitinated HIF-1 α . Since the HDACI-triggered degradation can be blocked by proteasome inhibitors, but not by lysosomal inhibitors, it is clear that the proteasome system is required [18]. Based on the above facts, it is clear that HDACIs induce HIF-1 α destruction by a ubiquitination-independent proteasome system (UIPS), whereas the precise mechanism remains to be dissected.

6.3. Is Hsp90 the Major Player in HDACI-Triggered Degradation of HIF-1 α ? Studies with Hsp90 inhibitors reveal a good candidate which may be responsible for HDAC-triggered degradation of HIF-1 α . Hsp90 is known to associate with nonnative structures of many proteins and is responsible for protein folding in general [153]. Hsp90 has three functional domains, the ATP binding domain, protein binding domain, and dimerization domain. The normal function of Hsp90 depends on its ATPase activity because it is the principal binding site for drugs that target this protein [154]. Hsp90 inhibitors have also been explored as antitumor drugs [155]. A quick comparison reveals some obvious similarities between these two groups of drugs. (1) Both HDACI and HSP90 inhibitors have been reported to destabilize various mutated HSP90 client proteins in cells; (2) both groups of drugs enhance the levels of HSP70; (3) both groups of drugs decrease client proteins' interaction with HSP90 but increase its interaction with HSP70; (4) while HDACIs apparently inhibit deacetylases, since HSP90 function requires HDAC6 activity to maintain its deacetylated states [156–160], HDACIs function as HSP90 inhibitors indirectly (Figure 4).

It has been reported by independent laboratories that molecular chaperones including HSP70 and HSP90 directly interact with HIF-1 α , suggesting that HIF-1 α is one of the client proteins of the HSP machinery. Similar to HDACIs, the Hsp90 inhibitor 17-AGG triggers ubiquitination independent degradation of HIF-1 α [17, 18]. We noted that most of the reported protein degradation cases triggered by either HDACI or HSP90 inhibitor were observed in cells with normal ubiquitination system. So even though the proteins subjective to the drug-induced degradation were generally associated with ubiquitination, there is no real evidence to support that ubiquitination is an absolute prerequisite for their degradation.

6.4. Potential Role of α -Tubulin Acetylation in HDACIs-Mediated Degradation of HIF-1 α . Another possible acetylated protein that may play a role in HDACI-induced destabilization of HIF-1 α is the α subunit of tubulin heterodimers (α -tubulin). α -tubulin is an important component for the

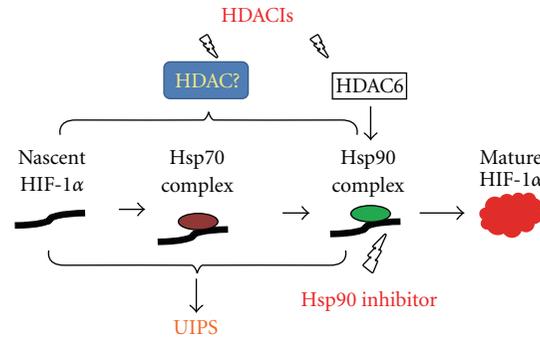


FIGURE 4: Acetylated Proteins Potentially Involved in the Control of HIF-1 α Maturation and Stability. HDACIs may lead to hyperacetylation of one or more proteins involved in the folding process of HIF-1 α . Similar to HSP90 inhibition-caused blockage of normal folding and mature processes of HIF-1 α , unfolded or misfolded HIF-1 α remains interacting with HSP70, which may eventually trigger a ubiquitination-independent degradation process.

formation of microtubules and other cellular structures with a variety of functions. The acetylation of α -tubulin was discovered long before the identification of histone deacetylase and protein acetyl transferases [161]. Its acetylation at Lys40 is a marker of stabilized microtubules [41, 43, 162] and is regulated by HDAC6 [163]. In addition, HDAC6 provides a link between protein acetylation and ubiquitination [164], suggesting a role in regulating protein stability. Since a role of HDAC6 in HIF-1 α stability has been proposed [18, 41, 137], one possibility is that microtubule dynamics may somehow be required for HIF-1 α stabilization. This hypothesis is supported by evidence that the small molecules disrupting the dynamics of microtubules also destabilize HIF1 α [113, 114, 165]. However, how and why microtubule dynamics affects HIF-1 α stability remains unknown.

7. The Acetylases and Deacetylases Involved in HIF Function

Because of the complexity and possible functional redundancy, it may be difficult to identify an individual member that is exclusively responsible for the regulation of HIF acetylation and function. As discussed above, the role of hARD1 in HIF-1 α acetylation is controversial. A role of HDAC7 in regulating HIF-1 function was first proposed, based on its interaction with HIF-1 α but not HIF-2 α [117]. HDAC7 was found to increase the transactivation activity of HIF-1, and it is thought to be a transactivation coactivator of HIF-1 [117]. So far several Class II HDACs have been proposed to regulate HIF-1 α stability [116]. However, since HDAC7 does not interact with HIF-2 α , it cannot be used to fully explain the repressive effects of HDACIs on HIF-2 α CAD. It is shown that HDAC4 and HDAC6 coimmunoprecipitated with HIF-1 α and the specific inhibition of HDAC4 and HDAC6 repress HIF-1 α stability [116]. It is possible that multiple deacetylases are involved in HDACI-induced modulation of HIF function, and that different cell types, different physiological conditions or signaling pathways may implicate different HDACs in the regulation of HIF function.

8. Conclusions and Perspective

The above discussion is based on experimental evidence and published literature that may link the biochemical effects of HDACIs to the repression of HIF function. The discussions are generally focused on deacetylases, acetylation substrates, and their potential relevance to the regulation of HIF function. It is clear that the transcription complexes of HIF-1 and HIF-2 require an activity of type I/II deacetylase for their transactivation activity. This deacetylase-dependent transactivation represents a unique feature of HIF function. It is also conclusive that higher doses of HDACIs induce the degradation of HIF-1 α through a proteasome-dependent pathway. This degradation can be mediated by an ubiquitination-independent mechanism. We expect further investigation in this field would bring new insight into the molecular and biochemical mechanisms underlying the anti-HIF and antiangiogenic effects of inhibitors of type I/II HDACs. It is also important to point out that a member of the class III HDACs, Sirt1, has been reported to deacetylate HIF-1 α and HIF-2 α and repress HIF- α activity [150, 166], further showing the complexity of effect of acetylation on HIF function. A thorough understanding of the regulation of HIF- α by protein acetylation is essential for future exploration aiming to modulate HIF function *in vivo* by targeting HDACs.

While it is conclusive that in addition to serving as epigenetic therapeutics, the inhibitors for class I and II deacetylases also repress HIF function, the underlying mechanisms remain far from clear. A better understanding of the mechanisms may be beneficial not only for better efficacy of cancer therapy, but also for prevention of side effects to normal organs. Particularly, given the large number of deacetylases and their important roles in transcriptional regulation, epigenetic programming, chromosomal remodeling, and other cellular processes, it is possible that nonselectively blocking deacetylases may cause unpredictable side effects. Obviously, a nonexhaustive list of imminent future directions should include (1) identifying the acetylases and deacetylases involved in HIF function under defined conditions in specific cell types, (2) identifying the HIF regulatory

factors subjective to acetylation, (3) defining the specific acetylation sites of their substrates and their relevance to HIF- α , (4) defining the upstream signaling pathways that regulate HIF function through protein acetylation. At least, a recent elegant study has linked cellular metabolic to protein acetylation [167].

Finally, because it is more difficult to destroy HIF-1 α protein, understanding the molecular basis of HDACI-mediated repression of HIF- α transactivation activity is expected to elucidate novel ways to repress HIF- α transactivation potential, regardless of its protein levels or stability. Considering that HIF function is required for the maintenance of oxygen and nutrient supply and for prevention of cell death under hypoxic conditions, global repression of HIF- α activity in the entire body, particularly for long-term use, may affect chronic adaptation required for ischemic disorders. On the other hand, since HIF function and dysregulated expression of VEGF play roles in tissue damage caused by ischemia-reperfusion, HDACI-mediated repression of HIF may prove to be beneficial for acute ischemia [168]. The effects of HDACIs on endothelial, bone marrow, neuronal, and circulatory systems warrant a thorough interrogation [169, 170]. Taken the potential adverse effects into consideration, a lesion-specific activation of prodrugs, which can be either HDACIs or compounds specifically regulating HIF function, may become an exciting exploration.

Abbreviations

CAD:	Carboxyl-terminal transactivation domain
CHIP:	Carboxyl terminus of HSP70-interacting protein
FDA:	Food and Drug Administration (USA)
FIH:	Factor inhibiting HIF-1
HAT:	Histone acetyltransferase
HADC:	Histone deacetylase
HDACI:	Histone deacetylase inhibitor
HIF:	Hypoxia-inducible factors
HSP:	Heat shock protein
HUPS:	Hydroxylation-ubiquitination-proteasomal system
NAD:	Amino-terminal transactivation domain
ODD:	Oxygen-dependent degradation domain
PHD:	Prolyl hydroxylases
TAP:	Transactivation potential
UIPS:	Ubiquitination independent proteasomal system
VEGF:	vascular endothelial growth factor.

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Review Article

Physical and Functional HAT/HDAC Interplay Regulates Protein Acetylation Balance

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The balance between protein acetylation and deacetylation controls several physiological and pathological cellular processes, and the enzymes involved in the maintenance of this equilibrium—acetyltransferases (HATs) and deacetylases (HDACs)—have been widely studied. Presently, the evidences obtained in this field suggest that the dynamic acetylation equilibrium is mostly maintained through the physical and functional interplay between HAT and HDAC activities. This model overcomes the classical vision in which the epigenetic marks of acetylation have only an activating function whereas deacetylation marks have a repressing activity. Given the existence of several players involved in the preservation of this equilibrium, the identification of these complex networks of interacting proteins will likely foster our understanding of how cells regulate intracellular processes and respond to the extracellular environment and will offer the rationale for new therapeutic approaches based on epigenetic drugs in human diseases.

1. Introduction

Lysine acetylation is a reversible and highly regulated post-translational modification discovered on histones in 1968 [1], but the enzymes responsible for acetyl group addition to or removal from target proteins, known as histone acetyltransferases (HATs) and deacetylases (HDACs), respectively, had not been identified until 1995 [2]. In the past decade, the knowledge about this modification has grown exponentially with targets rapidly expanding from histones to transcription factors and other proteins such as metabolic enzymes and signaling regulators in the cytoplasm. Thus, lysine acetylation has emerged as a major posttranslational protein modification rivaling phosphorylation.

Numerous protein properties are regulated through lysine acetylation, including DNA-protein interaction, subcellular localization, transcriptional activity, stability, and involvement in signaling pathways [3–5]. Besides, the dynamic state of posttranslational protein acetylation is intimately linked to aging and to several major diseases such as cancer, retroviral pathogenesis, neurodegenerative disorders, and cardiovascular diseases [6–8].

At the chromatin level, it has been widely demonstrated that the balance between acetylation and deacetylation of histone and nonhistone proteins plays a pivotal role in the regulation of gene expression. The general model of transcription is based on the interaction among RNA Pol II, general transcription factors, coactivators, corepressors, and sequence-specific DNA-binding proteins (DBPs) [9–11], which confer tissue and signal-dependent specificity. Coactivator and corepressor complexes contain a variety of chromatin-modifying enzymes, including HATs and HDACs.

HATs are classified into two groups, HAT A and HAT B, depending on the mechanism of catalysis and on cellular localization. The members of the HAT A family are found in the nucleus, where they transfer the acetyl group from Acetyl-CoA to an ϵ -NH₂ group of histone N-tails after the assembly into nucleosomes. The HAT A family can be further divided into three subclasses depending on the homology with yeast proteins. Conversely, the members of the HAT B family act in the cytoplasm and transfer the acetyl group from Acetyl-CoA to an ϵ -NH₂ group of free histones prior to their deposition on the DNA (Table 1).

TABLE 1: Mammalian Members of HAT Family.

Mammalian HATs					
Class	Subclass	Homology to yeast	Mammalian members	Mechanism of catalysis	Cell localization
A	GNAT-family	Gcn5	GCN5L PCAF	Transfer of acetyl group from acetyl-CoA to ϵ -NH ₂ group of histone N-tails after the assembly into nucleosomes	Nucleus
	MYST-family	Esal; Sas2; Sas3	Tip60 HBO1 MORF MOZ CLOCK NCOAT MOF		
	Others	HAT1; Elp3; Hpa2; NutI	p300/CBP TFIIIC complex ACTR/SRC-I ATF-2		
B		Hat1	HAT1	Transfer of acetyl group from acetyl-CoA to ϵ -NH ₂ group of free histones prior to their deposition on DNA	Cytoplasm

HDACs can be grouped into four classes in relation to their phylogenetic conservation [12]. Class I, class II, and class IV, which are related to the yeast Rpd3, Had1, and Hos3 proteins, respectively, encompass the classical family of zinc-dependent HDACs, while class III consists of the NAD⁺-dependent yeast Sir2 homologues, which comprise the sirtuin family [13, 14] (Table 2).

In eukaryotes, HATs and HDACs are involved in several aspects of cellular homeostasis. For example, in yeast, the HAT Gcn5 is required for the regulation of various cellular processes such as cell response to stress, meiosis, and DNA replication [15–17]. In mammals, the HAT p300/CBP plays a pivotal role in cell growth, myotube differentiation, and apoptosis [18–20]. Additionally, PCAF, an HAT enzyme originally identified as a p300/CBP-binding protein, is known to play a key role in regulating myofilament contractile activity, the myogenic program, and adipocyte proliferation [21, 22]. The G1-S phase progression in the cell cycle is mediated by class I HDACs; homologous recombination involves members of the sirtuin family; members of HDACs are found in complexes with transcriptional repressors in multipotent neural progenitor; HDACs play a role in the prevention of cytotoxicity arising from protein aggregation in neural cells [23–29].

In the last years, various studies showed that HATs and HDACs are both targeted to the transcribed regions of active genes marked by phosphorylated RNA Pol II. These data give further complexity to the general model of gene expression, suggesting that the dynamic cycle of acetylation and deacetylation by the transient binding of HATs and HDACs may poise primed genes for future activation [19, 30, 31].

The present knowledge in this field suggests that the balance between acetyltransferases and deacetylases provides a major contribution to the regulation of cellular functions. Given the key role of this equilibrium in cell physiology and considering that it is lost in various pathological conditions, targeting acetyltransferases and/or deacetylases might represent an effective therapeutic approach for human diseases.

2. Histone Targets of HATs and HDACs: Epigenetic Regulation

Histone modifications, together with factors responsible for adding, interpreting, and removing epigenetic marks, regulate specific responses of the eukaryote genome, and this represents the basis of the “histone code hypothesis.” Indeed, epigenetic marks are sites of recognition for specific readers and effectors. In the case of acetylation marks, certain modified lysines represent specific binding surfaces for bromodomain-containing proteins, which are part of large complexes controlling chromatin architecture. Singular or combinatorial histone modifications impact on chromatin organization and structure. Well-studied examples of this mechanism are the contribution of H4K16 acetylation to the regulation of chromatin structure and the interaction between nonhistone proteins and chromatin fibers [32, 33]. Bromodomain-containing proteins represent a large class of chromatin-associated factors with at least 75 members expressed in humans [34]. Some of them have been identified as part of chromatin-remodeling complexes [35]. Indeed, acetylation of histones H3K4 and H3K14 plays a central role in the recruitment of SWI/SNF chromatin-remodeling

complexes and of the general transcription factor TFIID during transcription initiation [36].

Recent studies have proposed various regulatory mechanisms for histone acetylation and deacetylation on gene activity. Several evidences have shown that HAT or HDAC enzymes are stepwise recruited to a specific locus by various types of transcription factors. For example, *in vivo* experiments have revealed the existence of different kinetics for the accumulation of different components of SWI/SNF remodeling and SAGA-containing HAT complexes at a condensed chromatin locus [37]. In yeast, studies conducted on various genes indicated that multiple chromatin regulators are recruited in a temporal order [38, 39] and that the recruitment of HATs or HDACs depends on the kind of factors involved in the transcriptional program [40].

Other works suggest that these two enzymatic activities are both present simultaneously on the regulatory regions of target genes, and the transcription activation or repression depends on the activation of different pathways and/or the type of enzymes which stabilize these interactions. In mammals, the association of HATs and HDACs in the same complex has been demonstrated to support transcriptional competence during myogenesis and p53-dependent transcription. Based on these data, we have previously proposed an experimental model in which a deacetylase activity is recruited by the C/H3 region of p300 antagonizing p300 functionality [19].

The hypothesis that acetylating and deacetylating enzymes bind simultaneously to regulatory loci also arises from observations on the effects produced by deacetylase inhibitors, which cause general and local histone hyperacetylation in yeast and mammalian cells [41–43]. In human pancreatic and breast cancer cells, the expression of the TGF β type II receptor gene (*T β RRII*) is mediated by modulation of the components present in multiprotein complexes that bind to its promoter. Both the p300 and PCAF acetyltransferases and the HDAC1 deacetylase are potential components of these complexes, and treatment of cells with HDAC inhibitors leads to the recruitment of PCAF and p300, resulting in the activation of the *T β RRII* promoter and in the decrease of the amount of HDAC1 associated to the complexes [41, 44]. Besides, a very recent genomewide analysis carried out in yeast showed that the acetyltransferase Gcn5 colocalizes with one or more HDACs both in ORFs (open reading frames) and IGRs (intergenic regions). Moreover, Gcn5 binds significantly to ORF regions that are hyperacetylated on histones H3K9 and H3K14, which are Gcn5 substrates. In these loci, Gcn5 collaborates antagonistically with the class II histone deacetylase Clr3 to modulate acetylation levels and transcriptional elongation. These data suggest the existence of a functional link between HATs and HDACs in regulating the balance of histone acetylation [45]. Due to the similarity between yeast HATs and HDACs and mammalian complexes, these results are likely to be relevant also in mammals.

Taken together, these data support an epigenetic model in which the activity of HATs and HDACs and the position of acetylated or deacetylated histones within genes play a major role in gene regulation.

3. Nonhistone Targets of HATs and HDACs: The Acetylome

The cellular and physiological functions of lysine acetylation are not limited to the regulation of gene expression. Lysine acetylation assumes a wider significance in many physiological processes, as it also targets nonhistone proteins. Following the identification of additional localizations of HATs and HDACs in other cell compartments, a search for new targets has begun with the aim of determining potential novel biologic functions of these enzymes.

A proteomic analysis of lysine acetylation has identified 388 acetylation sites in 195 proteins derived from HeLa cells and mouse liver mitochondria proving a potential link between acetylation and mitochondrial function. Among nonhistone proteins, the authors found RNA splicing factors (HnRNPA1), chaperones (Hsp70, Hsp27, and Hsp90), structural proteins (actin and tropomyosin), signaling proteins (phospholipase *C β* 1 and annessin V) and also proteins involved in energy metabolism, and longevity-related mitochondrial proteins [4]. These new targets for the activity of HATs and HDACs comprise the so-called “Acetylome”.

Additionally, in a very recent work, high-resolution mass spectrometry was used to identify new lysine acetylation sites and evaluate acetylome changes after the inhibition of HDACs [4]. In this study, Choudhary and colleagues used the SILAC (stable-isotope labeling by amino acid in cell culture) technology coupled with an LTQ Orbitrap mass spectrometer. By labeling cellular proteomes with isotopes of different molecular weight, SILAC allows simultaneous quantification of specific acetylated peptides of mixed proteomes prepared under different experimental conditions with a reported false-discovery rate of only 0.1 to 0.3%. This strategy revealed that the acetylation pattern is conserved in cells derived from different tissue types and that acetylation preferentially targets large macromolecular complexes involved in several major nuclear processes, such as cell cycle-associated chromatin remodeling (SWI-SNF and methyltransferases complexes), protein turnover (the BRE1A and BRE1B ubiquitin ligases, the USP14 and Ubch37 deubiquitylases), and DNA damage and repair (phosphoinositide 3 kinase-related protein kinases [PIKKs]). In addition, since HDACs are common targets in cancer and neurodegenerative diseases, the authors characterized acetylome changes in response to HDACs inhibition at a global level. Using two different inhibitors (suberoylanilide hydroxamic acid [SAHA] and MS-275), they showed that the increase in acetylation was not equal in all histone sites and nonhistone proteins. This observation suggests that the activity of these inhibitors is highly specific to particular HDAC members; thus, a global understanding of these processes could reveal an unexpected clinical specificity of HDACs inhibitors.

Several evidences suggest that the acetylation balance is also very important for cell viability. Indeed, it has been shown that this balance (I) controls the stability of various proteins such as p53 [46], β -catenin [47], and SMAD7 [48], thereby modulating the signaling pathways in which these proteins are involved [49, 50]; (II) plays a

TABLE 2: Mammalian Members of HDAC Family.

Mammalian HDACs				
Class	Homology to yeast	Mammalian members	Mechanism of catalysis	Cell localization
I	Rpd3	HDAC1	Zn ²⁺ ion dependent	Ubiquitous
		HDAC2		
		HDAC3		
		HDAC8		
II	Hda1	HDAC4	Zn ²⁺ ion dependent	Shuttle between nucleus and cytoplasm
		HDAC5		
		HDAC7		
		HDAC9		
		HDAC6		
III	Sir2	SIRT1	NAD ⁺ dependent	Nucleus
		SIRT2		Cytoplasm
		SIRT3		Mitochondria
		SIRT4		Mitochondria
		SIRT5		Mitochondria
		SIRT6		Nucleus
		SIRT7		Nucleus
IV	HOS3	HDAC11	Zn ²⁺ ion dependent	Nucleus

key role in DNA replication, recombination, and repair by regulating the stability of WRN, a multifunctional protein responsible for these processes [51]; (III) regulates proteins involved in nucleocytoplasmic shuttling, such as Importin α , or in translocation to the nucleus [52]; (IV) suppresses toxic protein aggregation through the interaction between HDACs (HDAC4, HDAC6, SIRT2) and the members of a subclass of the DNAJB family or members of heat shock proteins (HSP90) which are known to counteract protein misfolding and aggregation associated with cytotoxicity and what is mentioned in [27, 53–55].

As observed for histone acetylation/deacetylation, the dynamic balance in the acetylation of nonhistone proteins seems to be maintained by a physical and functional interplay between HAT and HDAC activities. Indeed, we reported that deacetylase inhibitors (DIs) could enhance the autoacetylation activity of p300 immunoprecipitated from nuclear extracts, but not that of the same purified recombinant enzyme [19], indicating that the presence of HDAC in p300 multiprotein complexes could also affect nonhistone targets. Subsequently, several studies have identified a similar mechanism for other members of the HAT and HDAC families. An example is the interaction between p300 and Sirt2, for which a model has been proposed stating that p300 indirectly increases the transcriptional activity of p53 through acetylation and subsequent attenuation of the deacetylase function of Sirt2. The existence of this interaction network suggests that the transcriptional activation mediated by the p300 coactivator is not regulated solely through epigenetic modification of histones and transcription factors. Indeed, the direct interplay between the opposing enzymatic activities of HATs and HDACs also seems to play a nodal role in this model, and their ability to regulate

each other's activity appears involved in the control of common targets. In support of this hypothesis, it has been demonstrated that acetylation of Sirt2 by p300 attenuates α -tubulin deacetylation by Sirt2 [56]. Consistent with this idea, a recent study has shown that p300 can inactivate HDAC6 affecting its ability to interact with other signaling modulators [57]. One of these is Hsp90, whose interaction with HDAC6 is functional to the regulation of chaperone-dependent activation of the glucocorticoid receptor [58]. Taken together, these data imply a new function for p300 and other members of the HAT family, which is opposed to their well-characterized positive regulatory effect, suggesting that they can also play a negative regulatory role on target proteins.

4. Acetylation Balance at the Crossroad of Cell Proliferation and Differentiation

The maintenance of an undifferentiated state requires that chromatin architecture sustains the silencing of target genes involved in lineage progression. This implies an acetylation balance strongly shifted towards deacetylation. The opposite occurs during lineage progression, when these genes need to be activated, and thus the balance must be weighted towards acetylation. Any modification of this equilibrium will interfere with the proper execution of the proliferating/differentiating program and may contribute to the development of a pathologic condition. Hence, HATs and HDACs play a pivotal role in the differentiation/proliferation balance of several cells and tissues.

As described above, several studies conducted to decipher histone acetylation and deacetylation dynamics suggested

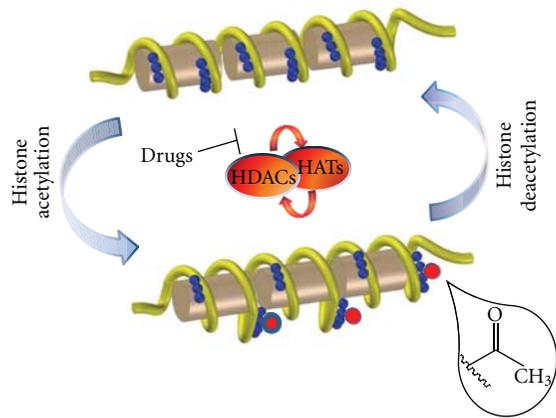


FIGURE 1: Physical and Functional interaction between HATs and HDACs regulates gene expression; HATs and HDACs enzymes are simultaneously present on the regulatory regions of target genes, and their opposing activities play a pivotal role for transcriptional competence. The use of selective HDAC inhibitors allows to restore the acetylation balance lost in several pathological conditions.

that the simultaneous presence of HATs and HDACs and their physical interaction play a key role in the regulation of the acetylation balance [59–61] (Figure 1). Increasing evidences indicate that the interaction between HATs and HDACs occurs in a dynamic fashion depending on the physiological conditions of the cell. Thus, acetylation homeostasis has to be considered intimately linked to cell homeostasis, and global changes in epigenetic modulators are important in the genetic reprogramming during cell proliferation or differentiation. Several examples of the role of writers and eraser of acetylation marks in these processes have been unveiled. Gcn5 and HDAC1 form a complex in mammalian cells, and their dynamic interaction is influenced by physiological processes such as cell differentiation. Indeed, treatment with TPA (phorbol ester tetradecanoyl phorbol acetate), which is known to induce differentiation, causes the replacement of Gcn5 with PCAF [59].

The interplay between HDACs and HATs is also linked to adipocyte differentiation. Downregulation of HDAC1 activity results in preferential histone hyperacetylation at the promoter regions of adipocyte marker genes. Specifically, HDAC1 directly interacts with PPAR γ , the master adipogenic factor, and represses its transcriptional activity. Thus, the downregulation of HDAC1 promotes PPAR γ activity by relieving it from repression. A very similar mechanism occurs during osteoblast differentiation suggesting that the modulation of HDAC expression and activity may be a general way of regulating cell differentiation [62].

Additionally, a recent work showed that neuronal outgrowth is driven by intrinsic and extrinsic factors ultimately affecting the balance between HAT and HDAC activities. Indeed, the addition of TSA leads to hyperacetylation of specific proneuronal outgrowth gene promoters. This suggests the presence of a positive feedback loop initiated by the relative increase in acetyltransferase activity through HDAC inhibition. This leads to histone hyperacetylation and activation of the CBP, p300, and PCAF promoters. p300/CBP

and PCAF in turn promote p53 acetylation which plays a key role in neuronal outgrowth [63].

The genetic reprogramming driving neuronal and oligodendrocyte lineage progression depends on the interplay between pluripotency-associated factors and epigenetic modulators. Thus, the acetylation balance plays a pivotal role in this process together with the histone trimethylation pattern. Several works showed that adult multipotent neural progenitor cells differentiated predominantly into neurons in the presence of the HDAC inhibitor valproic acid (VPA). VPA treatment also actively suppressed glial differentiation, even in conditions favoring lineage-specific differentiation [64, 65]. Moreover, the progressive restriction of cell lineage during differentiation from multipotent neural stem cells to oligodendrocyte progenitors (OPCs) is characterized by the progressive decrease of genes such as Sox2 (pluripotency-associated factor) and chromatin modifications on astrocytic and neuronal genes that are initiated by the activity of HDACs and are antagonized by Brca1 and Brm [66, 67]. The alteration of the HAT/HDAC balance can revert committed progenitors to multipotent cells displaying Sox2 expression [68].

A very recent study proposed a critical role in the differentiation of neural precursor cells for MRG15, a chromodomain-containing nuclear protein. The authors found that Mrg15-deficient neuronal precursor cells exhibit differentiation defects in addition to growth defects, suggesting the presence of a common pathway for HAT/HDAC activity modulation [69]. Interestingly, MRG15 associates in complexes both with the HAT Tip60 and with mSin3 and HDACs [70–75].

Besides, HDACs and HATs are also implicated in the regulation of E2F-responsive genes that control cell cycle progression. These genes are repressed by the coordinated activity of HDAC and the retinoblastoma protein, whose association requires the recruitment of HAT-TRRAP (an ATM-related protein) [76].

The simultaneous presence of acetyltransferases and deacetylases on regulatory regions of certain genes might explain the rapid changes occurring in promoter acetylation that drive the regulation of genes whose expression fluctuates rapidly (e.g., p21). This hypothesis arises from our recent work in which we showed the contribution of HDAC-HAT interaction to MyoD- and p53-dependent transcription [19]. The myogenic program is mediated by the MRF family of transcription factors—MyoD, Myf-5, myogenin, and MRF-4—which act sequentially to regulate the expression of genes involved in the early phase of determination and in the late differentiation phase. MyoD, the best studied MRF, is regulated by a dynamic flow of acetylation and deacetylation that influences its DNA binding ability [77–80].

How does this HAT-HDAC flow work? We have proposed a model in which transcriptional competence is conferred by the physical interaction between the MyoD transcription factor and HATs or HDACs [19]. In precommitted myoblasts, MyoD is expressed but inactive, because it is complexed with HDACs [78, 81]. The replacement of HDAC1 by PCAF helps MyoD, to drive differentiation by conferring transcriptional competence [19, 79]. Indeed, PCAF acetylates MyoD and

the acetyl marks mediate the recruitment of a bromodomain protein such as p300/CBP. Then, PCAF and p300/CBP coordinately acetylate lysine residues in the N-terminal tails of nucleosomal histones [82–85]. We have recently completed the picture by identifying the two signals that influence the composition of the muscle-specific transcriptome: p38MAPK, which is required for the recruitment of SWI-SNF, and Akt, which is involved in HDAC displacement and HAT recruitment and function [80].

The equilibrium between HATs and HDACs is also a nodal point in cell proliferation processes. It is well known that p300/CBP are involved in cell cycle control by regulating the transition from the G1 to the S phase. Indeed, cells lacking p300 activity display proliferation defects [86, 87]. The HAT activity of p300 is regulated by an intricate network of interactions between sumoylation and deacetylation epigenetic marks. A domain named CRD1 (Cell Cycle Regulatory Domain 1) consisting of two tandem SUMO modification sites has been found in p300/CBP proteins. The addition of SUMO to this domain is necessary for HDAC6 recruitment, thus promoting the transcriptional repressor activity of p300 [81]. This interaction mechanism might explain how a single enzymatic activity can both activate and repress transcription.

As described above, the reciprocal interplay between HATs and HDACs regulates various physiological cell processes; thus identification of the actors involved in the preservation of their equilibrium is highly desirable.

5. Epigenetic Drugs: A Matter of Acetylation Balance

The epigenetic etiology of many human diseases has led to the development of “epigenetic” therapies. As discussed above, the acetylation balance of chromatin regulates cell determination and cell fate suggesting that epigenetic drugs could prove useful for the treatment of muscle diseases, neurodegenerative disorders, and cancer. In the last decade, we provided several evidences *in vitro* and *in vivo* indicating that DIs could be a valid tool for pharmacological interventions in muscle dystrophies [19, 42, 88–90].

Deregulation of the equilibrium between HATs and HDACs has also been detected in several cancer types. The first evidences on how this balance is compromised in cancer cells derive from studies on the pathogenesis of leukemias [7].

In acute myeloid leukemia (AML) cells, various HDAC inhibitors have been used—including valproic acid (VPA), benzamide derivative (MS275), and suberoylanide hydroxamic acid (SAHA)—showing an anticancer action mediated by the expression of the tumor death ligand TRAIL and p21 [91]. In acute promyelocytic leukaemia (APL), which is characterized by chromosomal rearrangements leading to fusion proteins that involve the retinoic acid receptor (RAR), the fusion proteins maintain the ability to bind genes responsive to retinoic acid (RA), while exerting a modified biological function. In normal cells, the physiological concentration of RA induces the displacement of HDACs from RARs leading to their replacement with HATs at RA-regulated genes. In

APL cells, the physiological concentration of RA is not sufficient to achieve this effect. In this case, the simultaneous treatment with RA and HDAC inhibitors is efficient in restoring the correct activation of RA target genes [92].

At present, a promising strategy to reverse aberrant epigenetic changes associated with cancer is based on the use of HDAC inhibitors. Indeed, it has been demonstrated that HDAC inhibition induces proliferation arrest, differentiation, and apoptosis of cancer cells in culture and in animal models [93, 94].

The involvement of several enzymatic activities in cell transformation has stimulated the development of combinatory therapies. Cancer is characterized by the loss of cell cycle check points, and recent studies have identified an important cross talk between proteins involved in the cell cycle regulatory apparatus (Cdks) and proteins regulating histone acetylation. This observation suggests that the combined therapy with agents targeting both the acetylation balance and the Cdks might prove effective [95].

The treatment with HDAC inhibitors is beneficial also in B-cell lymphomas, in which the pathogenesis is caused by the deregulation of the BCL6 proto-oncogene. BCL6 is negatively regulated by p300 acetylation, which disrupts its ability to recruit HDACs, and it has been shown that pharmacological inhibition of HDAC activity causes the accumulation of the inactive acetylated form of BCL6 leading to cell cycle arrest and apoptosis of B-cell lymphoma cells [96].

DIs are a potential arm also in neurodegenerative disorders. Indeed, recent works have revealed that inhibition of HDACs ameliorates the cognitive and motor deficits characteristic of Huntington’s, Parkinson’s, and Alzheimer’s diseases (HD, PD, and AD). A common theme in these neurodegenerative disorders is the concept that intraneuronal aggregates such as plaques interfere with transcription and cause deficits in plasticity and cognition [97]. Therefore, if these aggregates interact with HAT/HDAC complexes, it might be possible to use epigenetic drugs for countering degeneration. For example, in PD α -synuclein mutated proteins aggregate in the nucleus and inhibit HAT-mediated acetyltransferase activity, thereby promoting neurotoxicity. In this case, HDAC inhibition is able to rescue α -synuclein-induced toxicity *in vivo* or *in vitro* [98]. In HD, nuclear translocation of mutated huntingtin proteins enhances ubiquitination and degradation of CBP through proteasome activity [99]. Neurodegeneration-coupled HAT activity loss is a molecular event that also characterizes AD; in this condition, the Presenilin1-dependent epsilon-cleavage product N-Cad/CTF2 binds to CBP and facilitates its proteasomal degradation [100].

Several regulating pathways, biological targets, and/or interactors of HATs/HDACs have been identified to date. This knowledge might be taken advantage of to develop therapeutic strategies based on the use of HDAC inhibitors in conjunction with other agents to obtain synergistic results.

6. Conclusion

The identification of a large number of acetylated targets has uncovered new players involved in the acetylation balance.

Nevertheless, it must be considered that acetyltransferases and deacetylases act primarily in protein complexes containing multiple cofactors and other enzymes responsible for a variety of posttranslational modifications and that cell processes are driven by the coordinated action of such complexes. The presence of one or another enzyme in a multiprotein complex is determined by signaling pathways activated by different external stimuli. Thus, a better understanding of the players involved in the response to these stimuli might allow specific pharmacological interventions aimed at preserving the physiological equilibrium of acetylation.

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Review Article

Roles and Targets of Class I and IIa Histone Deacetylases in Cardiac Hypertrophy

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Cardiac hypertrophy occurs in association with heart diseases and ultimately results in cardiac dysfunction and heart failure. Histone deacetylases (HDACs) are post-translational modifying enzymes that can deacetylate histones and non-histone proteins. Research with HDAC inhibitors has provided evidence that the class I HDACs are pro-hypertrophic. Among the class I HDACs, HDAC2 is activated by hypertrophic stresses in association with the induction of heat shock protein 70. Activated HDAC2 triggers hypertrophy by inhibiting the signal cascades of either Krüppel like factor 4 (KLF4) or inositol polyphosphate-5-phosphatase f (Inpp5f). Thus, modulators of HDAC2 enzymes, such as selective HDAC inhibitors, are considered to be an important target for heart diseases, especially for preventing cardiac hypertrophy. In contrast, class IIa HDACs have been shown to repress cardiac hypertrophy by inhibiting cardiac-specific transcription factors such as myocyte enhancer factor 2 (MEF2), GATA4, and NFAT in the heart. Studies of class IIa HDACs have shown that the underlying mechanism is regulated by nucleo-cytoplasm shuttling in response to a variety of stress signals. In this review, we focus on the class I and IIa HDACs that play critical roles in mediating cardiac hypertrophy and discuss the non-histone targets of HDACs in heart disease.

1. Introduction

Cardiac hypertrophy is an adaptive response to an initial exogenous hypertrophic stimulus that leads to a maladaptive state when the stress is prolonged [1]. Cardiac hypertrophy is characterized by increased cell size, enhanced protein synthesis, and heightened organization of the sarcomere. In this state, fetal genes, such as natriuretic peptide precursor type A (*Nppa*), myosin heavy polypeptide 7 (*Myh7*), and skeletal alpha-actin, are reactivated whereas cardiac contractile proteins like myosin heavy polypeptide 6 (*Myh6*) and calcium-handling proteins are repressed [2]. In addition, immediate-early genes encoding *c-fos*, *c-jun*, and heat shock proteins are upregulated [3, 4]. In humans, stresses such as chronic hypertension and myocardial infarction can trigger the heart to undergo remodeling processes characterized by myocyte hypertrophy, myocyte death, and fibrosis, often resulting in pathological heart diseases including reduced

cardiac function, cardiomyopathy, and heart failure [5–8]. Although it is believed that hypertrophy can be an adaptive response to exogenous stresses, advanced cardiac hypertrophy itself is associated with an increased risk of morbidity and mortality [9, 10].

Histones have long N-terminal tails and are subject to diverse post-translational modifications such as acetylation, methylation, phosphorylation, ubiquitination, and sumoylation. A well-characterized post-translational modification is acetylation, which occurs at the ϵ amino groups of lysine residues in the core histone. Acetylation of chromatin plays a central role in the epigenetic regulation of gene expression in eukaryotic cells. Acetylation is regulated by two opposing families of proteins, histone acetyltransferase (HAT), and histone deacetylases (HDACs). Recent evidence has indicated that different HDACs participate in a variety of heart diseases, such as arrhythmia, heart failure, and acute coronary syndromes, as well as in cardiac hypertrophy [11–19].

In mammals, there are four major classes of HDACs. Class I HDACs (HDAC1, 2, 3, and 8) are widely expressed and consist mainly of a catalytic domain. Class II HDACs are divided into two subclasses, IIa (HDAC4, 5, 7, and 9) and IIb (HDAC6 and 10). Class III HDACs are NAD(+)-dependent and are referred to as sirtuins (SIRT1-7). Most class IIa HDACs show cell-type-restricted expression patterns. Although many HDACs have a highly conserved domain, recent studies show that class I and IIa HDACs have opposing roles in regulating cardiac hypertrophy, and evidence for the mechanisms by which the distinct classes of HDACs act to control cardiac hypertrophy is growing. In this paper, we focus on the pathophysiological roles of class I and IIa HDACs in cardiac hypertrophy.

2. Heart Diseases Regulated by Class I HDACs: Cardiac Growth, Proliferation, Differentiation, Fibrosis, Ischemic Heart Disease, and Arrhythmia

HDACs are implicated as a regulator in various pathological heart diseases such as fibrosis, arrhythmia, ischemic heart diseases, and heart failure. Cardiac arrhythmia is related to a variety of cardiac stressors such as ischemia and an increase in wall stress. It is also associated with the renin-angiotensin-aldosterone system. A recent study indicated that the HDAC inhibitor, TSA, inhibits atrial fibrosis and arrhythmic inducibility and partially normalizes connexin 40 expression without changes in the angiotensin level in the Hopx transgenic mouse cardiac hypertrophy model [12].

Our group and others have demonstrated that myocardial fibrosis is reduced by HDAC inhibitors such as TSA and sodium valproate either in mice with left ventricular hypertrophy induced by aortic banding or in rats with right ventricular hypertrophy induced by pulmonary artery banding [15, 20, 21]. In addition, chemical HDAC inhibition was shown to reduce infarct size and improve ventricular function recovery in a model of myocardial ischemia and reperfusion injury, which suggests a novel therapeutic target for acute coronary syndromes [16, 17]. Sustained cardiac hypertrophic stimuli may lead to cardiomyopathy and heart failure. Likewise, heart failure with high mortality was prevented by apicidin derivatives with class I HDAC specificity in mice with heart failure induced by thoracic aortic constriction [13].

We [14] and other research groups [15, 20, 22] reported that class I and II broad HDAC inhibitors could prevent cardiac hypertrophy in animal models. We demonstrated that class I HDACs are required for the hypertrophic response in aortic banding or angiotensin II infusion-induced hypertrophy animal models with class I HDAC-selective HDAC inhibitor. Chemical HDAC inhibitors such as TSA or valproate induced the partial regression of pre-established cardiac hypertrophy. We were the first to show that class I HDACs may play a pro-hypertrophic role in the heart. Recently, another group reported similar results that broad-spectrum HDAC inhibitors such as TSA or scriptaid blunt the cardiac hypertrophy induced by aortic banding [15].

In rat neonatal cardiomyocytes, HDAC inhibition by TSA was also reported to blunt a stress-induced hypertrophic marker [22]. In addition, our group reported that sodium valproate, an alternate HDAC inhibitor, prevents right ventricular hypertrophy induced by pulmonary artery banding in rats [21]. Considering that class II HDACs work as anti-hypertrophic mediators, prevention of cardiac hypertrophy with nonspecific HDAC inhibitors strongly suggests that class I HDAC may function as a pro-hypertrophic regulator in the heart. These suggestions would be further supported by the reports that class I HDAC-selective inhibitors still show anti-hypertrophic effects [13, 14].

Recent studies have indicated that the class I HDACs (HDAC1, 2, 3, and 8) are involved in the control of cardiac growth, proliferation, and differentiation. Table 1 summarizes the phenotype and function of HDAC1, HDAC2, and HDAC3 in the heart. HDAC1 was reported to be a regulator of cardiomyogenesis, cell proliferation, and embryonic development. During cardiomyogenesis, downregulation of HDAC1 promotes the expression of Nkx2.5, a critical regulator of cardiac gene expression and heart development [23]. Disruption of the HDAC1 gene results in embryonic death before E10.5 as the result of severe proliferation defects [24]. These data suggest that HDAC1 is an important mediator in cardiac differentiation and early embryonic development.

Recent evidence implicates HDAC2 as a positive regulator of the hypertrophic response during embryonic development and in the adult heart. For example, HDAC2-deficient mice generated by a gene-trap technique show partial postnatal lethality, increased numbers of mitochondria, and abnormal sarcomere structure [26]. In addition, loss of HDAC2 makes the heart resistant to hypertrophic stimuli through the increased expression of inositol polyphosphate-5-phosphatase f (*Inpp5f*). The increased expression of *Inpp5f* is associated with activation of glycogen synthase kinase 3 β (GSK3 β). In contrast, transgenic mice overexpressing HDAC2 in the heart are sensitive to hypertrophic stimuli [26].

However, another group recently reported a different finding that HDAC2-null mice generated by use of the Cre/loxP system die within 24 hours after birth [25]. The most interesting difference between the studies of Trivedi et al. [26] and Montgomery et al. is that HDAC2 deletion by lacZ insertional mutation removes the region with exons 9–14, in the C-terminus of HDAC2 whereas the conditional deletion regions of HDAC2 include exons 2–4. Although HDAC2 consists mainly of the HDAC catalytic domain, the catalytic domain extends from partial exon 2 to exon 9. Therefore, the catalytic domain of HDAC2 seems to be implicated in postnatal viability. The different approaches to HDAC2 mutation resulted in distinct findings in response to hypertrophic stimuli such as chronic isoproterenol or aortic banding. For example, heart-specific deletion of HDAC2 did not result in an increased response to hypertrophic stimuli whereas HDAC2 disruption by lacZ insertional mutation prevented cardiac hypertrophy in response to hypertrophic stimuli [25].

HDAC3 is suggested to function as a regulator of cardiac myocyte proliferation during cardiac development

TABLE 1: The physiological role of class I and class IIa HDACs in cardiac development and heart diseases.

HDAC subtype	Model	Phenotype	Disease functions in the heart	References
HDAC1	P19CL16 cells	Differentiation	HDAC1 protein was decreased during cardiomyogenesis	Liu et al. [23]
	HDAC1-deficient mice	Proliferation	Embryo lethality because of proliferation defects	Lagger et al. [24], Montgomery et al. [25]
HDAC2	HDAC2 knockout mice	Proliferation	Proliferation rates of cardiac myocytes in HDAC2 knockout mice were elevated	Trivedi et al. [26]
	HDAC2 knockout mice	Proliferation	Increase in proliferation at P1 Lethality after P1	Montgomery et al. [25]
	HDAC2 knockout mice	Hypertrophy	HDAC2 knockout mice are resistant to cardiac hypertrophy	Trivedi et al. [26]
	HDAC2 transgenic mice	Hypertrophy	HDAC2 transgenic mice show cardiac hypertrophy	Trivedi et al. [26]
	Aortic banding mice Hsp70 knockout mice	Hypertrophy	HDAC2 and HSP70 cause cardiac hypertrophy	Kee et al. [14]
HDAC3	HDAC3 transgenic mice	Proliferation	HDAC3 transgenic mice show postnatal cardiac myocyte proliferation	Trivedi et al. [27]
	HDAC3 knockout mice	Hypertrophy	HDAC3 knockout mice show massive cardiac hypertrophy	Montgomery et al. [28]
	HDAC3 knockout mice	Metabolism	HDAC3 regulates cardiac energy metabolism	Montgomery et al. [28]
HDAC4	HDAC4 knockout mice		HDAC4 knockout mice die perinatally because of abnormal chondrocyte hypertrophy	Vega et al. [29]
HDAC5	HDAC5 knockout mice	Hypertrophy	Mice lacking HDAC5 develop enlarged hearts in response to pressure overload	Chang et al. [30]
HDAC9	HDAC9 knockout mice	Hypertrophy	HDAC9 mutant mice develop cardiac hypertrophy	Zhang et al. [31]

[27]. For example, transgenic hearts with myocyte-specific overexpression of HDAC3 show thickening in the ventricular wall and interventricular septum and an increase in phospho-histone H3 staining at neonatal day 1 (P1). The mRNA expression of several cyclin-dependent kinase inhibitors (p21, p27, p57, p16, and p15) is repressed in HDAC3 transgenic mice at P1. However, at 2 months of age, HDAC3 transgenic mice do not show an increase in the proliferation index. The results of this study imply that HDAC3 is related to cardiomyocyte proliferation rather than to cardiac hypertrophy. Recently, another group demonstrated a different role and phenotype of HDAC3 in the heart. In that study, although global deletion of HDAC3 resulted in embryonic death, heart-specific deletion of HDAC3 resulted in severe cardiac hypertrophy and increased expression of mitochondrial uncoupling genes at 3 months of age, and the mice survived until 4 months of age [28]. HDAC3 knockout mice elicited a significant increase in p21 expression [28].

Although HDACs have enzymatic activity to remove acetyl groups from protein, their existence alone is also important, because they can interact with numerous binding partners including transcription factors to form large complexes. For example, the enzyme activity and binding to MEF2 of class IIa HDACs play a critical role in a variety of diseases. In particular, MITR, a splice variant of HDAC9

that lacks a deacetylase domain, is known to have anti-hypertrophic action [31]. In this regard, studies should be taken with caution to determine which mechanism of either existence or activity is critical for the biological actions of HDACs. Our groups have demonstrated that the enzymatic activation of HDAC2 preceded the substantial hypertrophic phenotype in response to hypertrophic stimuli whereas the mutant form of HDAC2 did not result in a substantial hypertrophic phenotype. These results suggest that HDAC2 activation rather than its presence is important to induce cardiac hypertrophy [32].

Like the class IIa HDACs, the activities of the class I HDACs are regulated by post-translational modifications. The activity of HDAC1, HDAC2, and HDAC3 is mainly regulated by the phosphorylation of the casein kinase CK2 [33–35]. HDAC8 is phosphorylated by PKA [36]. For a better understanding of pathological heart diseases, further experiments are necessary to determine whether the phosphorylation status of the HDACs is modulated by phosphatases.

In summary, although both are class I HDACs, HDAC2 and HDAC3 have different functions in the regulation of cardiac hypertrophy and proliferation. HDAC2 works to cause cardiac hypertrophy accompanied by a repression of proliferation whereas HDAC3 acts to inhibit cardiac hypertrophy with increasing proliferation. We discuss the

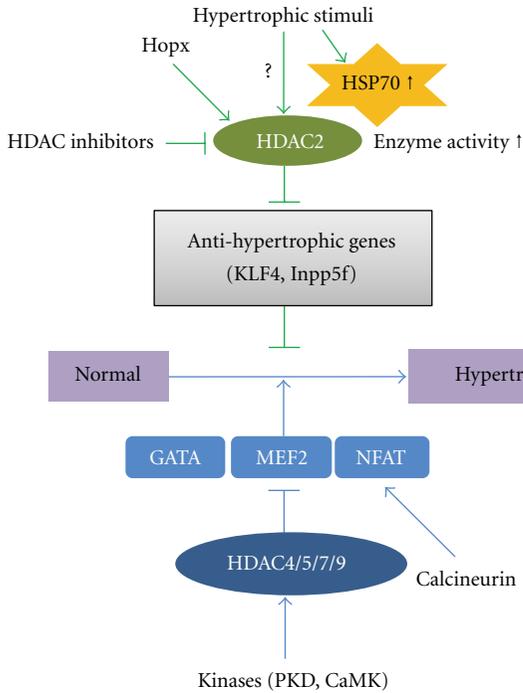


FIGURE 1: The signaling cascade of class I and IIa HDACs in the development of cardiac hypertrophy. Class I HDACs can be regulated by the Hopx/HDAC2/HSP70/KLF4 pathway. Cardiac hypertrophy is induced in Hopx transgenic mice through the recruitment of HDAC2. Hypertrophic stresses including pressure overload or agonist-induced HSP70 expression are followed by its association with HDAC2. The resultant increase in HDAC2 activity decreases the expression of anti-hypertrophic mediators including KLF4 and Inpp5f. This diagram is adapted from the works of Kook et al. [20] and Kee et al. [32, 38] (JCI, 2003; Circulation Research, 2008; JMCC, 2009). Class II HDACs can be activated by PKD or CaMK. They interact with MEF2. MEF2 also associates with GATA4 and NFAT transcription factor. NFAT can be triggered by calcineurin. Class IIa HDACs can repress cardiac hypertrophy through MEF2 either directly or indirectly.

mechanism by which HDAC2 regulates cardiac hypertrophy further in the next section.

3. HDAC2, a Class I HDAC, in Cardiac Hypertrophy

Recently, by use of HDAC2 knockout mice or transgenic mice and cardiomyocytes, our group and others have identified that HDAC2, a class I HDAC, positively regulates cardiac hypertrophy. Figure 1 shows the Hopx/HDAC2/HSP70/KLF4 signal cascades that regulate prohypertrophy pathways in the heart. Our hypothesis is supported by our previous study with transgenic Hopx mice. Hopx, a cardiac-specific regulator of gene transcription, is an unusual homeodomain protein that is expressed in embryonic and postnatal cardiac myocytes. Inactivation of Hopx causes severe developmental cardiac defects with cardiac proliferation. Hopx transgenic mice exhibit cardiac hypertrophy and cardiac fibrosis resulting from recruitment of HDAC2 whereas Hopx mutants do

not develop hypertrophy or recruit HDAC2. The cardiac hypertrophy induced in Hopx transgenic mice can be prevented by HDAC inhibitors such as TSA and valproate [20, 37]. Although previous studies showed an increase in HDAC activity in Hopx transgenic mice compared with wild-type mice, little is known about changes in the activity of each of the HDACs in cardiac hypertrophy. Thus, our group focused on determining HDAC activity in response to hypertrophic stimuli among the class I HDACs. We observed that only HDAC2 activity was induced in response to hypertrophic stimuli.

Heat shock proteins (HSPs) are induced by hypertrophic stimuli [3]. Thus, we determined whether the induction of HSPs in response to hypertrophic stresses induces cardiac hypertrophy. We found that the novel HDAC2-interacting partner HSP70 activates HDAC2 and induces cardiac hypertrophy. HDAC2 increased the cardiomyocyte cell size. In addition, cardiac hypertrophy and HDAC2 activation were blunted in HSP70 knockout mice [32]. These data strongly suggested that the HSP70/HDAC2 axis is an important mediator in the regulation of cardiac hypertrophy and highlighted HSP70 as a regulator of HDAC2 activity in response to hypertrophic stresses.

We next investigated whether HDAC2 could regulate downstream targets in cardiomyocytes. In the cardiomyocytes, Krüppel-like factor 4 (KLF4) was shown to be a target of HDAC2 and to repress *Nppa*, leading to the inhibition of cardiac hypertrophy [38]. KLF4 overexpression blocked the increase in *Nppa* transcript levels and [³H]-leucine incorporation induced by 20% FBS. In contrast, KLF4 knockdown stimulated hypertrophic phenotypes. The results of this study suggested that KLF4 is a novel anti-hypertrophic regulator. Recently, Liao et al. reported that KLF4 functions as a negative regulator of cardiac hypertrophy by use of the cardiomyocyte-specific KLF4 knockout mice [39].

Like KLF4, Inpp5f is known to be a downstream target of HDAC2 in mediating cardiac hypertrophy. HDAC2-regulation of Inpp5f is mediated through the phosphatidylinositol 3-kinase- (PI3K-) Akt-Gsk3 β pathway, which is important for growth control in heart. Overexpression of HDAC2 suppresses *Inpp5f* expression, and accumulation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) leads to the recruitment of protein kinase Akt. Activated Akt inactivates Gsk3 β by its phosphorylation. Suppression of Gsk3 β activity relieves the inhibition of some hypertrophic signaling pathways, such as c-Myc, GATA4, and β -catenin, leading to cardiac hypertrophy [26]. In the case of HDAC2 deficiency, the decrease in Akt activity and increase in Gsk3 β activity result in resistance to cardiac hypertrophy. Furthermore, hypertrophic responsiveness was shown to be reduced in mice with *Inpp5f* overexpression whereas knockout mice showed augmented hypertrophy [40].

4. Negative Regulation of Cardiac Hypertrophy and Heart Failure by Class IIa HDACs

The class IIa HDACs (HDAC4, HDAC5, HDAC7, and HDAC9) are expressed in muscles and brain and have an

N-terminal regulatory domain that mediates interactions with transcription factors, coactivators, and corepressors [41–43]. The underlying mechanism and functional role of the class IIa HDACs in hypertrophy has been intensively investigated by the Olson group by use of knockout mice. Diverse hypertrophic stimuli lead to the activation of cardiac HDAC kinases such as protein kinase C (PKC), protein kinase D (PKD), and calcium/calmodulin-dependent protein kinase (CaMK). In this section, we describe how each of the class II HDACs regulates and affects cardiac hypertrophy through signaling pathways and associated proteins.

HDAC4 is phosphorylated by activated CaMKII in response to phenylephrine. Phosphorylated HDAC4 is translocated into the cytoplasm by 14-3-3 protein, resulting in derepression of the MEF2 gene, which leads to hypertrophic growth [44]. HDAC4 appears to interact with and inhibit the activity of runt-related transcription factor 2 (Runx2). HDAC4-null mice show skeletal defects and chondrocyte hypertrophy, whereas overexpression of HDAC4 inhibits chondrocyte hypertrophy and shows Runx2 loss of function of phenotypes [29].

HDAC5, an alternate class IIa HDAC, has similar actions in regulating cardiac hypertrophy. Phosphorylation of HDAC5 by PKC or PKD leads this protein to bind to 14-3-3 protein, which stimulates the nuclear export of HDAC5. In cardiomyocytes, an HDAC5 phosphorylation mutant was shown to be resistant to the PKC signaling pathway and to block cardiomyocyte hypertrophy [45]. Chang et al. demonstrated that HDAC5 knockout mice develop cardiac hypertrophy with age in response to pressure overload and calcineurin signaling whereas overexpression of HDAC5 blocks hypertrophy [30]. Although little is known about the role of HDAC7 in cardiac hypertrophy, this HDAC is also regulated by shuttling [46]. By contrast, the role of HDAC9 has been well scrutinized in cardiac hypertrophy by use of knockout mice. Class IIa HDACs and MEF2-interacting transcriptional repressor (MITR), a splice variant of HDAC9, have MEF2 binding sites in the N-terminus and an HDAC catalytic domain in the C-terminal region. HDAC9 mutant mice show cardiac hypertrophy with advanced age in vivo and are hypersensitive to hypertrophic stimuli such as thoracic aortic banding [31]. HDAC5/9 double knockout mice show an even greater degree of cardiac hypertrophy than mice lacking either HDAC5 or HDAC9 [30]. In addition, other pro-hypertrophic transcription factors including GATA4 and NFAT have been shown to be repressed indirectly by class IIa HDACs [47]. These transcription factors have been shown to be associated with MEF2 [48]. Therefore, class IIa HDACs also can repress the activity of these cardiac-specific transcription factors through MEF2. Taken together, these findings suggest that the class IIa HDACs function as negative regulators of cardiac hypertrophy by repressing MEF2/GATA4/NFAT-mediated gene expression [31, 49].

Although most of the class IIa HDACs can be shuttled from the nucleus to the cytoplasm by phosphorylation in the control of cardiac hypertrophy [50, 51], recent evidence has shown that HDAC4 is subject to oxidative modification in the mediation of cardiac hypertrophy [52]. When HDAC4 oxidation is induced by hypertrophic stimuli, thioredoxin1,

a 12-kDa antioxidant, regulates the nucleocytoplasmic shuttling of HDAC4. In addition, cysteine residues in HDAC2 are subject to nitrosylation modification in neurons [53, 54]. Therefore, like phosphorylation, other forms of post-translational modification may be critical mechanisms in the regulation of cardiac hypertrophy.

5. Class III HDACs: Aging and Stress

Class III HDACs consist of seven Sirt isoforms (Sirt 1 to Sirt 7). The Sirts, known as sirtuins, are NAD(+)-dependent acetyl-lysine deacetylases. The Sirts have been shown to be involved in aging and protection against oxidative stress in the heart [55, 56]. Heart-specific moderate overexpression of Sirt 1 in mice results in the retardation of aging and a protective response to oxidative stress [57]. As for Sirt 1, similar results have been shown for Sirt 3 and Sirt7 in regulating oxidative stress-mediated cell death in cardiomyocytes [58, 59]. Considering the beneficial effects of Sirts, intensive studies of Sirt activators will enhance our understanding of the underlying mechanism in heart diseases. Sirt activators are being developed, and clinical trials have been conducted for some metabolic diseases such as diabetes [60]. We suggest that Sirt may be a critical target in mediating cardiovascular diseases.

6. Deacetylation of Nonhistone Proteins by HDACs: Transcription Factors and Cytoskeleton Proteins

Non-histone proteins have been identified as substrates for HDACs [61]. Many studies have demonstrated that different HDACs target various transcription factors in cancer cells, during protein repair, in immune reactions, and in redox regulation. For example, p53, the most commonly mutated gene in cancer cells, is deacetylated by HDAC1 and SIRT1 (Sir2 alpha) [62, 63]. SRY, a sex-determining region on the Y chromosome, is deacetylated by HDAC3 [64]. STAT3 (signal transducers and activators of transcription) is completely deacetylated by HDAC3 and, to a lesser extent, by HDAC1 and HDAC2 [65]. SHP, orphan nuclear receptor, recruits HDAC1, HDAC3, HDAC6, and SIRT1 [66, 67]. Currently, however, the HDACs responsible for the deacetylation of SHP are unknown. E2F1 transcription factor, which organizes early cell cycle progression, can be deacetylated by HDAC1 via an indirect interaction mediated by Rb [68]. RelA, a class II NF- κ B family member (nuclear factor kappa-light chain enhancer of activated B cells) that acts to regulate the immune response, can be deacetylated by HDAC3 and SIRT1 [69, 70]. Peroxiredoxins (I and II), Hsp90, and α -tubulin are a specific target of HDAC6 [71]. Ku70, a repair protein, is deacetylated by SIRT1 [72]. GCM (glial cell missing) transcription factor can be deacetylated by HDAC1, HDAC3, HDAC4, and HDAC5 [73].

Deacetylation of non-histone proteins can alter protein stability, localization, protein-protein interaction, DNA-binding affinity, and transcriptional activity. In addition,

deacetylation of non-histone proteins promotes ubiquitination and decreases the half-life of the protein [74]. Cardiac-specific transcription factors such as GATA4, NFAT, NF κ B, MEF2, SRF, Smads, Nkx2-5, YY1, Hand, Egr-1, and CREB are reported to be involved in heart diseases. Among these, some transcription factors may be a direct target of HDACs in the heart. For example, the MEF2D family of transcription factors, which mediate cardiac hypertrophy, is deacetylated by HDAC3 [75]. In addition, SIRT1, an NAD⁺-dependent deacetylase, induces the acetylation of MEF2 [51]. In contrast, although HDAC4 and HDAC5 are known partners of MEF2, they do not function as a MEF2 deacetylase. MyoD, a skeletal muscle-specific transcription factor, is deacetylated by HDAC1 and SIRT1 [76].

Among the six GATA transcription factors, GATA4 has been extensively studied as a regulator of cardiac development and differentiation as well as in hypertrophic growth in adult heart [77–79]. Many studies have indicated that GATAs are mainly acetylated by p300, PCAF, and CBP with HAT activity [80]. It has been reported that GATAs can interact with different HDACs although little is known about whether these GATAs are substrates of the HDACs. It is suggested that GATA4 may be deacetylated by class I and IIa HDACs because the acetylation of GATA4 is augmented by treatment with TSA [81].

Recent data show that the TGF β -Smad signaling pathway can regulate the development of cardiomyocyte hypertrophy and cardiac fibrosis. For example, Smad2 overexpression inhibits agonist-induced hypertrophy in culture, and Smad4-deficient mice develop cardiac hypertrophy. Lysine residues of Smad7, an inhibitory Smad, are deacetylated by SIRT1 (class III histone deacetylase) [82]. Smad7 overexpression suppresses collagen type I and III whereas decreased Smad7 expression in cardiac fibroblasts is found in the infarcted rat heart [83].

The homeobox transcription factor Nkx2.5 is also implicated in the hypertrophic response and in heart development. Little is known about the direct deacetylation of Nkx2.5 by HDACs. However, associated HDAC5 was found in a complex with acetylated Nkx2.5 in cardiomyocytes [84]. Thus, Nkx2.5 is expected to be a direct target of HDACs.

YY1 is a multifunctional transcription factor that can repress or activate the transcription of many genes. YY1 residues acetylated by p300 and PCAF are deacetylated by HDAC1 and HDAC2 in HeLa cells [85]. Recent evidence demonstrated that YY1 interacts with HDAC5 in cardiac myocytes and plays an anti-hypertrophic role in pathological hypertrophy [86]. Similarly, YY1 interacts with HDAC5 in differentiated H9c2 cells and functions as a regulator in muscle differentiation [87]. In addition, YY1 was shown to associate with HDAC2 and to activate the expression of BNP, which is a marker related to cardiac myocyte hypertrophy [88].

In addition to transcription factors, other non-histone proteins can be regulated by deacetylation. For example, α -tubulin, another marker of cardiac hypertrophy, is increased in a congestive heart failure animal model. α -Tubulin was found to be deacetylated by HDAC6 and SIRT2 [89–91].

In summary, the epigenetic modification of diverse proteins including heart-specific transcription factors can play vital roles in a variety of heart diseases. The finding of direct HDAC targets will provide us a new understanding of pathological heart diseases and insight into promising drug development for the treatment of cardiac hypertrophy.

7. Summary and Further Suggestions

The functional roles of the HDACs in heart development and in heart diseases including cardiac hypertrophy and heart failure have been reviewed. Class I and IIa HDACs have been shown to have opposing roles in the regulation of cardiac myocyte growth and pathological heart disease. HDAC2, a class I HDAC, induces cardiac hypertrophy in response to early hypertrophic stimuli whereas class IIa HDACs including HDAC4, HDAC5, and HDAC9 suppress cardiac hypertrophy. Several lines of evidence with HDAC inhibitors show that class I HDACs play a more important role than class IIa HDACs in cardiac hypertrophy. Recent work has demonstrated that broad-spectrum HDAC inhibitors as well as class I or II HDAC-specific inhibitors prevent cardiac hypertrophy, fibrosis, and ischemic heart diseases. Unlike class I and IIa HDACs, class III HDACs play an important role in regulating oxidative stress in heart diseases with metabolic syndromes. Future studies to develop the agonists of class III HDACs could be a good strategy for treating and protecting against the cardiovascular diseases associated with aging.

HDAC inhibitors are being highlighted as cancer therapy. SAHA, known as Vorinostat, has been approved by the Food and Drug Administration [92], and several other HDAC inhibitors are currently being investigated in clinical trials [93, 94]. Thus, one can easily assume that inhibition of HDAC may be applied for the treatment of cardiac diseases as well as various cancers. For example, if heart-specific HDAC inhibitors are developed and if the regulation of HDAC activity or HDAC-interacting protein interaction can be modulated by specific chemicals, the application of such drugs will be of great benefit. Actually, several pharmaceutical companies are developing chemicals to regulate HDAC itself or its target-specific protein to treat pathological diseases such as in heart and muscle.

Because of their involvement in many pathological heart diseases, further investigation of the HDACs is vital to understand the underlying mechanism of their action, their biological roles, and their direct substrates. The enzymatic activity of HDACs can be regulated by phosphorylation/dephosphorylation by protein kinases and phosphatases or by protein-protein interaction. Therefore, to improve our understanding of the mechanism by which HDAC2 acts in cardiac hypertrophy, we will need to search for HDAC2 kinase and other post-translational modifications such as sumoylation, ubiquitination, S-nitrosylation, carbonylation (alkylation), and glycosylation. In this regard, further studies are necessary to elucidate the mechanism and effect of novel epigenetic regulator-mediated cardiac diseases. Further experimental evidence should be provided to develop selective pharmacological drugs such as inhibitors of

pathology-associated HDACs or HDAC modifying enzyme to initiate clinical trials for the treatment of cardiac diseases. We must critically consider the non-histone targets of HDACs. These targets contain many important transcription factors. Knowledge from the targets of HDAC regulation is expected to improve our comprehension of HDAC biological activity and functions.

Further studies of both the downstream and the upstream targets of HDAC2 will extend our knowledge of the biological role of the HDACs and may suggest novel combined therapeutic strategies for heart diseases.

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Review Article

The Tale of Protein Lysine Acetylation in the Cytoplasm

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Reversible posttranslational modification of internal lysines in many cellular or viral proteins is now emerging as part of critical signalling processes controlling a variety of cellular functions beyond chromatin and transcription. This paper aims at demonstrating the role of lysine acetylation in the cytoplasm driving and coordinating key events such as cytoskeleton dynamics, intracellular trafficking, vesicle fusion, metabolism, and stress response.

1. Introduction

The story of cytoplasmic protein lysine acetylation begins long after the discovery of posttranslational acetylation of histones in the sixties [1]. In fact only in 1985 tubulin was described as the first acetylated cytoplasmic protein [2, 3]. Since then numerous other cytoplasmic proteins have been found acetylated. The first global proteomic study on acetylated proteins describes 37 acetylated proteins in the cytoplasmic fraction of Hela cells and 133 in mouse liver mitochondria [4]. In another study about 250 acetylated proteins, presumably localized in the cytoplasm, have been identified [5].

Lysine (K) acetylation is catalysed by a lysine acetyltransferase (KAT) formerly called histone acetyltransferase (HAT) (for new nomenclature see Allis et al. [6]), which transfers the acetyl-group of acetyl-CoA to the epsilon-amino group of an internal lysine residue. The reverse reaction is accomplished by deacetylases, which can be divided into several classes. The class I, IIa, IIb, and IV enzymes are zinc dependant, whereas members of the class III family (also called sirtuins) use NAD⁺ as a cofactor for the deacetylation reaction.

The high number of acetylated proteins present in the cytoplasm points to a critical role for this posttranslational modification in the regulation of cytoplasmic events. In this paper we will focus on selected examples illustrating the role of reversible acetylation in the cytoplasm and we will mention some proteins, identified by proteomic

approaches as being acetylated, when it could be important in the context of the discussed cellular processes. We will also provide an overview on what is known about the cytoplasmic localisation of the enzymes implicated in lysine (de)acetylation.

2. Cytoplasmic Localisation of KATs and HDACs

2.1. KATs. Most of the extensively characterised acetyltransferases are known as nuclear enzymes (see Table 1 for overview). Even Hat1, the first identified acetyltransferase, is predominantly localized in the nucleus, although it has been characterized as a type B acetyltransferase which refers to its role in the cytoplasm where it acetylates newly synthesised histones [7–9]. Under some circumstances, like early during development or in colorectal tumors, the cytoplasmic fraction of Hat1 increases [10, 11]. In addition, it has recently been shown that two different isoforms of Hat1 are expressed in keratinocytes, which differ in their cellular localisation [12].

Although acetyltransferases are considered mostly nuclear, an increasing number of studies reports on their nucleocytoplasmic transport. For instance PCAF and Gcn5 become phosphorylated following growth factor receptor signalling, which induces their translocation to the nucleus [13]. The cellular localisation of PCAF is not only regulated by phosphorylation. In fact PCAF can autoacetylate lysine

residues within its nuclear localisation signal (NLS) and deacetylation of these lysine residues leads to cytoplasmic accumulation of PCAF [14]. CBP and p300 behave almost like Hat1 since, during oocyte maturation, they are first found in the cytoplasm before being imported into the nucleus [15]. Furthermore, similar to Hat1, p300 is found in the cytoplasm in breast carcinomas but not in the adjacent normal mammary gland [16]. Both nuclear localisation and nuclear export signals have been found in Tip60, a member of the MYST family of acetyltransferases. Tip60 can be recruited to the plasma membrane by the amyloid precursor protein, which induces its phosphorylation and subsequent translocation to the nucleus [17]. In addition Tip60 appears in two splice variants. Whereas the longer isoform is essentially found in the nucleus, the shorter form, Tip60 beta (also called PLIP), missing exon 5, is located in both the cytoplasm and the nucleus and interacts with cytosolic phospholipase A2 [18, 19]. The acetyltransferase ATF2 also has nuclear localisation and export signals and is able to shuttle between the nucleus and the cytoplasm. Heterodimerisation with c-Jun in the nucleus is necessary to retain ATF2 in the nuclear compartment [20].

The most astonishing fact is that, although tubulin was the first acetylated protein described in the cytoplasm [2, 3] and a tubulin acetyltransferase activity had already been purified and characterised in 1986 [21], the scientific community had to wait until 2009 to put a name on an enzyme able to acetylate α -tubulin. In fact the acetyltransferase E1p3, which is the catalytic subunit of the transcriptional elongator complex, was found able to acetylate tubulin, which is essential for the maturation of cortical neurons [22]. The role of E1p3 as a tubulin acetyltransferase important for neuronal development has been confirmed in a genetic RNAi suppression screen for regulators of α -tubulin acetylation using the nematode *Caenorhabditis elegans* [23]. Besides its role in tubulin acetylation, E1p3 has been implicated in the regulation of other processes outside the nucleus such as stress signalling, tRNA modification, exocytosis and actin dynamics [24–28]. A study by Esberg et al., however, questions the role of E1p3 as an acetyltransferase since various effects observed in E1p3 deficient cells could be attributed to its role in tRNA modifications and more specifically to the absence of wobble uridine modified lysine and glutamine tRNA species [29, 30]. A recently published paper describes Gcn5 as another acetyltransferase, capable of acetylating α -tubulin after its recruitment to microtubules via a cytoplasmic proteolytic fragment of myc [31]. Importantly, another recent paper identifies Mec-17, a Gcn5-related protein, as a major α -tubulin acetyltransferase [32]. Finally, two reports suggest that enzymes having N-acetyltransferase activity could also acetylate internal lysine residues and contribute to tubulin acetylation [33, 34].

2.2. HDACs. Compared to the Hat families there are by far more examples of deacetylases located in the cytoplasm (see Table 2 for list of HDACs). Even members of the type I family of deacetylases, which in the past have been considered as strictly nuclear proteins, can be found in the

cytoplasm. For instance HDAC1 is a nuclear enzyme which, under pathological situations, is exported via CRM1 into the cytoplasm where it binds to kinesin motors, hindering cargo transport [35]. HDAC3, another type I deacetylase, is found in the nucleus and the cytoplasm and has both nuclear export as well as nuclear localisation signals [36]. It is retained in the cytoplasm via its interaction with I κ B α [37] until TNF- α signalling leads to the degradation of this binding partner [38]. Furthermore HDAC3 can associate with the plasma membrane where it is phosphorylated by src thereby increasing its activity [39]. Surprisingly, in contrast to the other class I enzymes which are more nuclear, HDAC8 is essentially in the cytoplasm where it associates with smooth muscle α actin to regulate cell contractility [40, 41].

Class IIa deacetylases (4, 5, 7 and 9) [42] are known to shuttle between the nucleus and the cytoplasm [43, 44]. HDAC7 is the only member of this family reported to be present also in mitochondria; it leaves mitochondria and the nucleus to accumulate in the cytoplasm after initiation of apoptosis [45]. All class IIa members have nuclear localisation signals and binding sites for proteins of the 14-3-3 family. An important regulatory mechanism relies on the phosphorylation of these 14-3-3 binding sites inducing the interaction with 14-3-3 proteins and subsequent accumulation in the cytoplasm. Cytoplasmic accumulation has been attributed to 14-3-3-mediated CRM1- dependant nuclear export but two publications have shown more recently that phosphorylation can target the NLS and that the deacetylases are retained in the cytoplasm after binding of 14-3-3 to the phosphorylated NLS and disruption of the nuclear import [46, 47]. Independent of its phosphorylation status, the nuclear export of HDAC4 is regulated by oxidation of two of its cysteine residues resulting in an intramolecular disulfide bridge, whose reduction inhibits nuclear export [48]. HDAC7 is another example of a class IIa enzyme, which can be exported from the nucleus independently of its phosphorylation status and 14-3-3 binding [49].

The translocation of type I and IIa HDACs into the cytoplasm in all the examples mentioned above (except HDAC8) is generally considered as a regulatory mechanism, which serves to reduce their action in the nucleus and so far cytoplasmic substrates have only been found for HDAC4 (as discussed below).

The most extensively studied cytoplasmic deacetylase is the type IIb enzyme HDAC6, which has several cytoplasmic substrates, including tubulin [50–52], cortactin [53], Hsp90 [54–57], β -catenin [58], and peroxiredoxin [59]. Although it bears intrinsic nuclear import and export signals, HDAC6 is almost exclusively localised in the cytoplasm [60, 61], where its actions underlie multiple regulatory processes. One way to regulate its action is to change its localisation within the cytoplasm. As mentioned below HDAC6 is translocated together with Hsp90 and Rac1 to membrane ruffles after PDGF stimulation where it influences actin dynamics [62]. Tubulin deacetylation by HDAC6 can be prevented by Cbl, due to competitive binding to β -tubulin [63]. Another way influencing its activity is the binding to various partner proteins either directly or indirectly. An example of an indirect interaction essential for HDAC6 activity is the formation

TABLE 1: Lysine (K) acetyltransferases.

New Name	Former name human	Former name D. melanogaster	Former name S. cerevisiae	Former name S. pombe	HAT complexes
GNAT family					
KAT1	HAT1	CG2051	Hat1	Hat1/Hag603	HAT-B
KAT2		dGCN5/PCAF	Gcn5	Gcn5	SAGA, ADA, ATAC
KAT2A	hGCN5				STAGA, TFTC
KAT2B	PCAF				PCAF complex
KAT9	ELP3	dELP3/CG15433	Elp3	Elp3	Elongator for RNA polymerase II
	HsMEC-17				
p300/CBP family					
KAT3		dCBP/NEJ			
KAT3A	CBP				
KAT3B	P300				
MYST family					
KAT5	TIP60/PLIP	dTIP60	Esa1	Mst1	NuA4
KAT6		(CG1894)	Sas3	(Mst2)	NuA3
KAT6A	MOZ/MYST3				
KAT6B	MORF/MYST4				
KAT7	HBO1/MYST2			(Mst2)	
KAT8	hMOF/MYST1	dMOF (CG1894)	Sas2	(Mst2)	MSL complex
nuclear receptor coactivators					
KAT13A	SRC-1				
KAT13B	ACTR				
	SRC-3				
	TIF-2				
	GRIP1				
	ATF-2				
Divers					
KAT4	TAF1 (TAFII250)	dTAF1	Taf1	Taf1	TFIID
KAT10			Hap2		
KAT11			Rtt109		
KAT12	TFIIIC90				
KAT13C	P160				
KAT13D	CLOCK				

of a tripartite complex composed of the farnesyltransferase, HDAC6, and microtubules. Disruption of the complex by KO of the alpha subunit of the farnesyltransferase or by an inhibitor of this enzyme leads to enhanced tubulin acetylation [64]. BBIP10, a protein essential for primary cilia formation also binds to and inhibits HDAC6, but so far it is not clear whether this interaction is direct or not [65]. An example of a direct interaction is the binding of Dia2 to one of the two deacetylase domains of HDAC6, which leads to activation of HDAC6 in osteoclasts [66]. Two other examples are the microtubule-associated protein tau and the tubulin polymerisation promoting protein TPPP/p25, which can bind to HDAC6 and inhibit its activity leading

to a subsequent increase in microtubule acetylation [67–69]. Ilp45 (a newly discovered protein) as well as CYLD, a deubiquitinating enzyme, can both inhibit HDAC6 by direct binding to the deacetylase domains of HDAC6, which in the case of Ilp45 will also lead to HDAC6 degradation [70, 71]. A third way to regulate HDAC6's activity is by its posttranslational modification. It has been shown for instance that HDAC6 interacts with EGFR and is phosphorylated by EGFR at Tyr570 after ligand-induced receptor activation [72]. This reduces HDAC6 activity leading to tubulin acetylation and accelerated delivery of endocytosed EGFR to lysosomes. In contrast Aurora A, a mitotic ser/thr kinase, phosphorylates HDAC6 leading to its activation

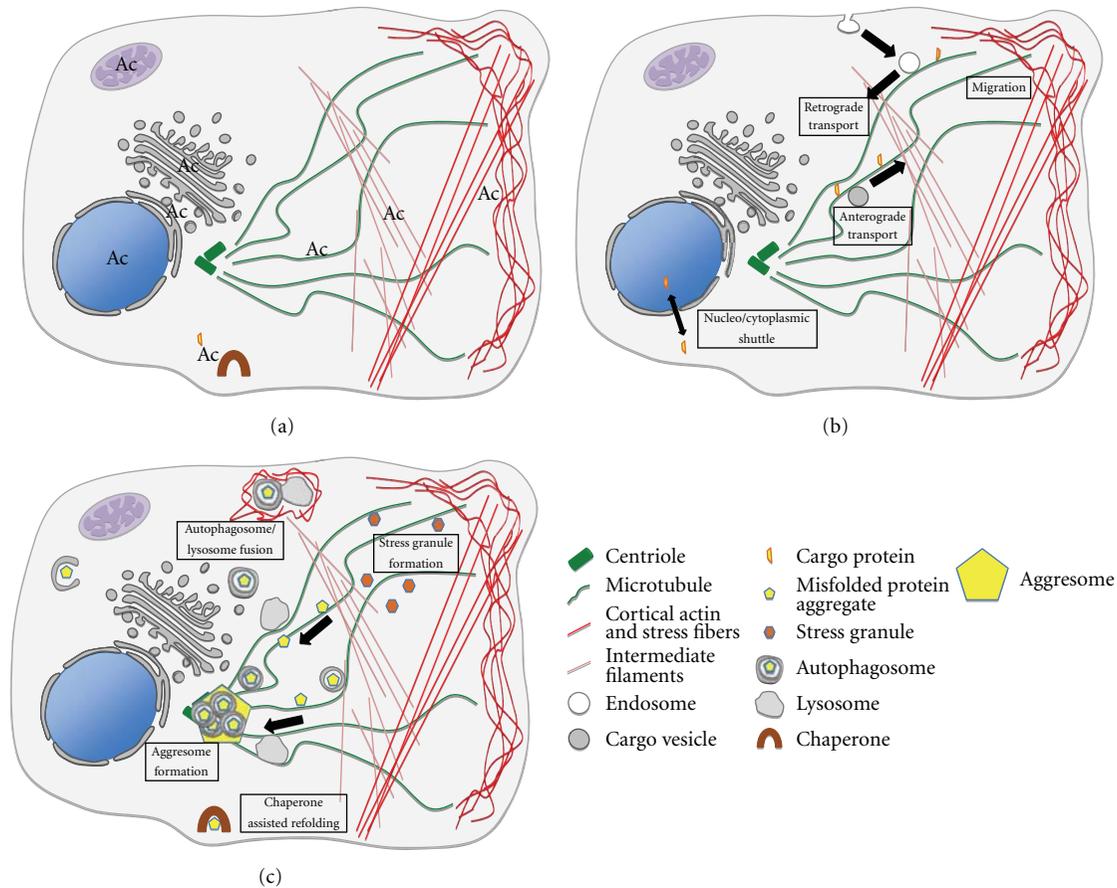


FIGURE 1: Schematic overview of cytoplasmic mechanisms regulated by acetylation. (a) Localisation of acetylation events within a cell. Indicated are individual acetylated proteins and cytoskeletal structures (cortical actin and actin stress fibers, microtubules, intermediate filaments) as well as organelles (nucleus, mitochondria, ER, ERGIC, golgi) in which acetylation and deacetylation events take place. (b) Involvement of reversible acetylation in the regulation of various cytoplasmic processes, including cell migration following cytoskeleton remodelling, individual protein transport as well as vesicular transport and nucleocytoplasmic shuttling of proteins. (c) Regulation of stress response and cytoplasmic cleanup systems by various acetylation/deacetylation events.

resulting in tubulin deacetylation and resorption of primary cilia in human retinal cells [73]. In this context it is worth mentioning that HDAC6 (as well as other HDACs) can be found in complexes together with phosphatases and both enzymes are active under these conditions [74]. HDAC6 activity, besides being regulated by phosphorylation, can also be reduced by p300-mediated acetylation [75]. In addition to its deacetylase function, HDAC6 has other important cellular tasks, such as cargo transport by binding to the dynein and kinesin motor complexes, or binding to mono and polyubiquitin, which is necessary for aggresome formation and induction of quality control autophagy (QC-autophagy) (for review see [76, 77]).

HDAC10, the other class IIb enzyme, is present in the cytoplasm and the nucleus and, in contrast to the class IIa family members, is not exported through a CRM1-mediated transport mechanism [78–81]. Finally HDAC11, the only type IV deacetylase, is essentially a nuclear enzyme but can be found in the cytoplasm in latent HIV infected cells [82, 83].

The sirtuin or class III family of deacetylases are a functionally distinct family for two reasons. First they

depend on the cofactor NAD^+ for their activity, and second they do not release free acetate after hydrolyses of the acetyl group but rather transfer it onto ADP-ribose. In mammals, seven sirtuins have been described (Sirt1-7) [84]. Only Sirt1, Sirt2, and Sirt3 have a true deacetylase activity all the others have only ADP-ribosyltransferase activity. At least, this was considered the truth until it was observed that Sirt6 could deacetylate lysine 9 of histone H3 [85]. No cytoplasmic localisation, however, has been observed for Sirt6; we will therefore focus only on Sirt1-3 [86].

Sirt1 has both a nuclear localisation and a CRM1-dependent export signal and, in most cells, it is present both in the nucleus and the cytoplasm [87]. Upon induction of neural differentiation it is transiently transported into the nucleus but for NGF-induced neurite outgrowth it has to be present in the cytoplasm [88, 89]. As discussed below, Sirt1 has important roles in the cytoplasm linked to autophagy and cell migration via deacetylation of cortactin [90, 91].

Sirt2 is almost only localised in the cytoplasm due to an active CRM1-dependent export mechanism [92]. It has been implicated in tubulin deacetylation [93] and seems to

TABLE 2: Lysine (K) deacetylases.

Name	Localisation	Cytoplasmic substrates
Class I		
HDAC1	mainly nucleus	
HDAC2	nucleus	
HDAC3	mainly nucleus/cytoplasm	
HDAC8	mainly cytoplasm	
class IIa		
HDAC4	cytoplasm/nucleus	MLP, DNAJB8
HDAC5	cytoplasm/nucleus	
HDAC7	cytoplasm/mitochondria/nucleus	
HDAC9	cytoplasm/nucleus	
Class IIb		
HDAC6	cytoplasm	tubulin, cortactin, Hsp90, β -catenin, peroxiredoxin
HDAC10	cytoplasm/nucleus	
Class IV		
HDAC11	mainly nucleus	
Class III(sirtuins)		
sirt1	cytoplasm/nucleus	cortactin, Atg5, Atg7, Atg8
sirt2	cytoplasm	tubulin, cortactin?
sirt3	mitochondria/long form cytoplasm	
sirt4	mitochondria	
sirt5	mitochondria	
sirt6	nucleus	
sirt7	nucleus	

deacetylate tubulin especially under certain circumstances, for instance during mitosis to regulate mitotic microtubule dynamics [94] or during oligodendrocyte differentiation [95]. It was suggested that Sirt2 inhibition could reduce alpha synuclein toxicity in Parkinson disease, but the molecular interplay has not been identified [96, 97].

Finally, Sirt3 is known as a mitochondrial enzyme (for a detailed review see [84]), but a long form of Sirt3 partially localised in the cytoplasm has been recently described [98].

At last, it is worth mentioning that quite a few nuclear and cytoplasmic acetylated proteins are targeted by both, class I/II as well as class III, deacetylases as for instance histones [99], tubulin [50–52, 93], Ku70 [98, 100], p53 [101, 102], and cortactin [53, 91]. For most of these examples it is not clear whether HDACs of different classes show a preference for particular acetylated lysine residues in these proteins. Actually, motif preferences for lysine acetylation only start to emerge. The local sequence context around

acetylated lysine residues in cytoplasmic proteins appears to be different from that of histones but similar to that of other nuclear proteins. Furthermore mitochondrial acetylation motifs differ from acetylation sites found in both histones and other nuclear/cytoplasmic proteins [4, 5]. In spite of these advances a precise consensus sequence for the substrate specificity of different Kats or HDACs has yet to be defined.

3. Functional Implications of Cytoplasmic Lysine Acetylation

3.1. Regulation of the Cytoskeleton. Since the cytoskeleton is involved in many cytoplasmic processes, we will start by describing what is known about the regulatory effect of acetylation on the three main types of cytoskeletal components, namely, actin filaments, microtubules, and intermediate filaments (Figures 1(a) and 1(b)).

3.1.1. Actin Filaments. The globular monomeric form of actin (G-actin) polymerises to form a double helix (filamentous or F-actin), which can then be bundled into stress fibers. There are three major isoforms of actin. Beta and gamma actin form the stress fibers are important for cell shape and cell movement. Alpha actin constitutes the microfilaments in muscle cells, which together with myosin assures the traction forces necessary not only for muscle contraction but also for cytoplasmic streaming in nonmuscle cells. Using proteomic approaches it has been shown that all three actin isoforms can be acetylated [4, 5] and acetylation of lysine 61 in gamma actin may result in stabilisation of actin stress fibers [4]. Not only actin itself but also several regulatory proteins of the actin cytoskeleton are modified by acetylation. For instance six out of the seven subunits of the Arp2/3 complex, which is important for actin nucleation, are acetylated [5]. Cortactin binds to F-actin in the cell cortex and can recruit the Arp2/3 complex to the cortical actin cytoskeleton when it has been activated by phosphorylation. But phosphorylation is not the only regulatory modification of cortactin. It has been shown that cortactin can be acetylated by p300 or PCAF on nine different lysine residues present in its central repeat domain [53, 91]. When acetylated its translocation to the cell periphery is inhibited and its actin binding capacity reduced. This leads to diminished actin dynamics at the cell periphery and thus to altered cell motility. Deacetylation of cortactin is mediated via HDAC6 and Sirt1 (maybe also Sirt2), which may act in cooperation to induce cell migration [53, 91]. In fact the actin cytoskeleton is essential for cell migration since it is necessary for the formation of membrane ruffles or lamellipodia, filopodia, and actin stress fibers, which reflect different dynamic states of the actin cytoskeleton. Actin dynamics is also regulated by small GTPases of the Rho family. A simplified general scheme implicates RhoA in stress fiber formation, Rac1 in lamellipodia and cdc42 in filopodia formation. The activation of these G-proteins in turn depends on the action of GDIs (GDP dissociation inhibitors), GAPs (GTPase-activating proteins), and GEFs (GDP/GTP exchange factors). It has been shown that acetylation of RhoGDI alpha prevents its inhibitory action

on Rho family members leading to enhanced stress fiber and filopodia formation [4]. RhoA can also be inhibited by the E-cadherin-binding protein p120 catenin. Acetylation of p120 changes its subcellular localisation thereby relieving its inhibitory action on RhoA [4]. Rac1 activation, leading to membrane ruffle formation, associated macropinocytosis, and cell migration, depends on deacetylation of the chaperon Hsp90 by HDAC6, but the precise sequence of events has not been elucidated [62].

Muscle contraction can also be regulated by acetylation as observed for actomyosin filament activity in cardiac muscle cells. In fact the muscle LIM protein (MLP), which colocalizes with myofilaments at the Z-disc of sarcomeres, is a mechanical stretch sensor. Acetylation of MLP by PCAF enhances the calcium sensitivity of myofilaments. HDAC4 seems to be the deacetylase responsible for MLP deacetylation [103].

3.1.2. Microtubules. As already mentioned, α -tubulin, which together with β -tubulin forms the heterodimeric building block of microtubules, was the first cytoplasmic protein described to be acetylated [2, 3]. α -tubulin is acetylated on lysine 40 which, until recently, was considered to be the only acetylated lysine residue in tubulin isoforms. Interestingly, in a recent systematic proteomic identification approach for acetylated proteins, lysine 40 of α -tubulin was not identified. This is most probably due to the fact that an immunoprecipitation step using a pan acetylated lysine antibody has been used in this approach, which most probably has a low affinity for this particular residue. However, several other acetylated lysine residues in different tubulin isoforms (α as well as β subunits) were detected, but their role and significance have still to be elucidated [5]. Even our understanding of the functional role of the acetylation at lysine 40 of α -tubulin is not clear cut. The reason for this may come from the fact that the expression of nonacetylatable α -tubulin does not result in any obvious phenotype [104–106], nor does hyperacetylation following KO of HDAC6 [107]. In general, acetylated microtubules are considered to represent the subpopulation of more stable microtubules in the cell. There has been however a long debate in the literature as to whether acetylation is the cause or a consequence of microtubule stabilisation. While it has been suggested that acetylation just accumulates with time in microtubules with a long half-life [108], others have proposed that acetylation could directly stabilise microtubules [50, 51]. Yet, hyperacetylation in neuronal cells does not lead to microtubule stabilisation [109]. Also, in cells KO for the tubulin deacetylase HDAC6, hyperacetylation of microtubules is not accompanied by detyrosination, which is the other hallmark of stable microtubules [62]. Thus we are proposing an alternative hypothesis for the role of tubulin acetylation. In fact, acetylated microtubules are not very abundant in most of the cells cultured *in vitro*. There are, however, a few exceptions. At least three cell types have heavily acetylated microtubules in their cytoplasm; these are neurons, platelets, and megacaryocytes, the platelet progenitors. In addition there are cellular substructures

formed by microtubules, which are also heavily acetylated like primary cilia, flagella, mitotic spindles and midbodies. What all these structures and cell types have in common is the need of microtubule bundles. Thus acetylation may allow for more efficient bundling of microtubules, which in turn may lead to enhanced stability of these microtubule bundles. In support of this hypothesis are several observations described in the literature. Naranatt et al. have shown that, 30 min after infection of cells by HHV-8, a transient increase of microtubule acetylation is seen which is accompanied by a thickening of microtubule bundles. Both, acetylation as well as the thickening of microtubules, come back to basal levels about 2-3 hours after infection [110]. Similarly, pneumolysin, a virulence factor of streptococcus pneumonia, can induce microtubule acetylation and concomitant microtubule bundling [111]. Also, overexpression of calpain 6, a catalytically inactive calpain isoform, induces hyperacetylation and bundling of microtubules [112]. Another example is the tubulin polymerisation promoting protein TPPP/p25, which induces not only tubulin polymerisation but also acetylation and subsequent bundling and stabilisation of microtubules [68]. Microtubule bundles are also promoted by p180, a rough ER associated protein, after induction of tubulin acetylation [113]. In addition, early during neuronal commitment an acetylated array of microtubules is formed which is arranged in a bundle of parallel microtubules [114].

Besides its contribution in stabilisation and/or bundling of microtubules, tubulin acetylation plays an important role in cellular transport events as discussed below. It should be mentioned here that there is a coordinated interplay between the actin cytoskeleton and the microtubular network of which one coordinator is the RhoA effector Dia [115, 116], which can bind to microtubules and, as mentioned above, also binds to actin filaments. In fact Dia has been shown to influence microtubule orientation, stability and acetylation states. Some of the studies conducted so far describe enhanced microtubule acetylation following RhoA and Dia activation [110, 117–119]. It is not known, however, whether in these cases enhanced acetylation is due to direct Dia mediated HDAC6 inhibition or to activation of a tubulin acetyltransferase. In another study using osteoclasts, Dia activation leads to the opposite effect namely deacetylation of microtubules due to the interaction of Dia with, and concomitant activation of, HDAC6 [66]. The outcome of Dia activation on microtubule acetylation/stabilisation may thus be cell type dependent. Further complexity might be added to the finetuning of these regulatory events by the fact that a Dia-related isoform seems to be acetylated itself [5].

3.1.3. Intermediate Filaments. At least some of the constituents of the third class of cytoskeletal elements, the intermediate filaments, have also been found acetylated. One example is vimentin, which is acetylated on several lysine residues [5], another is cytokeratin 8, which is acetylated on three lysine residues. In contrast to tubulin and actin however, in this case, acetylation seems to destabilize the polymer [120, 121].

3.2. Transport. Cellular transport along microtubule tracks is particularly important in cells, which have long extensions like axons and dendrites of neuronal cells. This may be the reason why the important role of microtubule acetylation for cargo transport has first been observed in neurons. The microtubule motor kinesin is implicated in anterograde transport, whereas dynein motors are involved in retrograde transfer of cellular materials (Figure 1(b)). It has been shown that binding of kinesin-1 to and mobility on microtubules is enhanced by tubulin acetylation and thus delivery of Jip-1 (a cargo protein of kinesin-1) to neurite tips is accelerated [122]. Another example of more efficient kinesin-1-mediated transport along acetylated microtubules is the vesicular transport of the neurotrophic factor BDNF. In neurons of Huntington disease patients, BDNF is not transported and secreted efficiently due to a polyglutamine expansion in the htt protein, which is unable to stimulate microtubule-based transport. Hyperacetylation of microtubules can rescue this deficiency. In addition the authors show that both kinesin as well as dynein-dependent microtubular transport is enhanced by tubulin acetylation [109]. Regulation of receptor trafficking by enhancing the speed of endosome transport is also augmented by microtubule acetylation [72, 123]. EGFR vesicle recycling however is not influenced under these conditions [123]. Collectively, these observations indicate that the transport of individual cargo proteins, as well as some but not all vesicular transport processes, are regulated by microtubule acetylation (Figure 1(b)).

Viruses have developed strategies to profit from the host cellular transport machinery for efficient infection. The herpes virus HHV-8 for instance is able to enhance microtubule acetylation by activation of RhoA and its effector Dia2, which speeds the dynein dependent delivery of viral DNA to the nucleus [110]. The adenovirus acts in a similar way [119]. The vaccinia virus instead inhibits RhoA and, in turn, Dia2 leading to diminished tubulin acetylation but increased tubulin dynamics in the cell periphery, which in this case could help viral release [117]. Efficient invasion of bladder cells by uropathogenic *E. coli* bacteria also requires HDAC6-mediated deacetylation of microtubules [124].

In the context of cellular transport mechanisms it is worth mentioning that several motor proteins have themselves been identified as being acetylated and future studies will have to tell whether transport regulation attributed to microtubule acetylation could have been influenced also by motor protein acetylation [5].

3.3. Translation, Quality Control, and Cytoplasmic Cleanup. Acetylation has been widely studied for its role in transcriptional regulation in the nucleus and it is almost astonishing that, to our knowledge, no observation on translational regulation by acetylation has been described so far. It is worth mentioning, however, that in global proteomic studies a huge number of translation initiation factors or ribosomal proteins have been found acetylated [4, 5]. Thus a possible role of acetylation in translational control mechanisms remains to be discovered. The amount of expressed proteins is not only controlled at the transcriptional and translational

levels but also by regulation of their half-life. Acetylation is widely used to influence protein stability in both directions but, since this topic has been reviewed recently [125], it will not be discussed here.

Besides quantity control, the cell is also equipped with quality control and cleanup systems for cellular proteins to avoid the accumulation of misfolded, aggregated proteins in the cytoplasm, which would have a deleterious effect on coordinated normal cellular functions (Figure 1(c)). Following translation many newly synthesised proteins have to be assisted for correct folding or assembly into multi-subunit complexes. This is ensured by chaperones. Chaperones are also implicated in the management of misfolded proteins which arise following different environmental stress conditions like heat shock, oxidative stress and aging, or pathological expression of proteins due to mutations or over-expression. In case of an unsuccessful management by chaperones, misfolded proteins are ubiquitinated and degraded by the proteasome. After a prolonged or extensive stress period or under experimental overexpression of proteins, the chaperone/proteasome pathway cannot cope anymore with the massive conformational defects of proteins, which accumulate and aggregate in the cytoplasm. This gives rise to a more global stress response, leading to stress granules and aggresome formation and induction of QC-autophagy. The important role of HDAC6 in all of these different cellular defence mechanisms has been well described (for review see [76, 77]) and will not be discussed in detail in this paper. We will rather emphasize the critical importance of some acetylated lysine residues within other key actors involved in these processes.

3.3.1. Chaperones/Proteasome. Chaperones are well known as proteins assisting nucleosome formation. There are however also several chaperones important for the folding or assembly of cytoplasmic protein complexes and many chaperones have been discovered following heat shock treatment, which leads not only to their transcriptional induction but also to their immediate activation. Hsp90 can be acetylated on several lysine residues and its activation state depends on its acetylation status. Its immediate activation is mediated by HDAC6-dependent deacetylation, which is essential for ATP binding of Hsp90 [54] and for its binding to its cochaperone p23 as well as to various client proteins like AhR (aryl hydrocarbon receptor) [126], Bcr-Abl, AKT, c-Raf [54], or the glucocorticoid receptor [57, 127]. Besides other acetylated lysine residues deacetylation of lysine 294 in Hsp90 is particularly important for cochaperone and client binding [56] but, as mentioned above, HDAC6 mediated deacetylation of this site is also responsible for Hsp90 mediated Rac1 activation resulting in actin reorganisation and cell migration [62]. The different functional roles of Hsp90 could be dictated by its cellular localisation since Hsp90 is translocated to membrane ruffles to mediate its role in actin remodelling [62]. By deacetylation of Hsp90, HDAC6 may regulate its own survival since HDAC6 has been shown to be itself an Hsp90 client protein [128].

Alpha A-crystallin, a chaperone in the soluble fraction of eye lenses, becomes acetylated on lysine 70, a region supposed to be important for its activity, and alphaB-crystallin appears to be acetylated on lysine 92 [129, 130]. Crystallins may thus be another example of chaperones whose activity is regulated by acetylation. The chaperone DNAJB8 can be acetylated on two conserved lysine residues situated in its carboxyterminal part. Although these lysine residues are not implicated in substrate binding, deacetylation mediated by HDAC4 activates the chaperone and suppresses cytotoxic protein aggregation [131].

As mentioned above, in case chaperones are unable to assist correct folding of their client proteins, these misfolded proteins are eliminated by proteasomal degradation. One example is again Hsp90. In its inactive ADP-bound state it associates with the cochaperone Hsp70, which prepares the misfolded client protein for degradation by its association with a ubiquitin ligase. Hsp70 is also hyperacetylated following either class I HDAC or HDAC6 inhibition. In contrast to Hsp90, hyperacetylation of Hsp70 promotes its interaction with client proteins leading to their ubiquitination and degradation by the proteasome [132].

3.3.2. Stress Granules/Aggresome/Autophagy. When the load of misfolded proteins is too high the cells suspend their normal function and respond with an immediate block of mRNA translation (except for stress response factors like heat shock proteins/chaperones). Accumulation of the halted transcripts gives rise to the appearance of stress granules. In parallel, the accumulating misfolded proteins become ubiquitinated and transported along microtubules to the centrosome where they form the so-called aggresome. HDAC6 is essential for both stress granule and aggresome formation [133, 134]. It has been suggested that HDAC6 could act in the transport process as an adaptor by binding to the dynein motor complex on the one hand and binding via its ubiquitin binding domain to ubiquitinated proteins on the other [133]. Parkin, a ubiquitin E3 ligase (often mutated in Parkinson disease), tightly binds to HDAC6 and is also transported by HDAC6 in a microtubule motor dependent manner to the forming aggresome. There may be ongoing parkin-mediated ubiquitination of misfolded proteins during transport along the microtubules [135, 136]. Given that microtubule motor-dependent transport is enhanced when microtubules are acetylated one would expect that the deacetylase activity of HDAC6 is inhibited during these transport processes and that deacetylase deficient forms of HDAC6 should be able to rescue stress granule and aggresome formation in HDAC6 KO cells. Since this is not the case and in addition, HDAC6 and parkin are both active during the transport to the aggresome, it seems that a deacetylation event is essential for aggresome and stress granule formation. Whether tubulin is the relevant substrate or not remains to be determined.

Aggresomes or smaller ubiquitinated misfolded protein aggregates are cleared by basal or QC-autophagy. Double membranous vesicular compartments called autophagosomes are formed in order to isolate the cytoplasm containing the material to be eliminated. These vesicles then

fuse with lysosomes, and the lysosomal enzymes ensure the proteolytic digestion of their content. HDAC6 is essential for the delivery of autophagosomal constituents and lysosomes to the pericentriolar localised aggresome by transport along microtubule tracts, as observed for the clearing of aggregated huntington [137]. HDAC6 then plays a second role in the fusion between autophagosomes and lysosomes by recruiting and deacetylating cortactin which in turn recruits actin filaments to tether the two vesicle populations [138, 139]. QC-autophagy can be distinguished from starvation-induced autophagy since the latter does not depend on HDAC6. Both, however, depend on the same autophagy-related genes (Atg). Recent studies have shown that starvation-induced autophagy is regulated by the acetylation status of Atg5, Atg7, and Atg8, which are proteins required for the formation of the autophagosome. They are acetylated by p300, which prevents autophagosome formation [140]. Deacetylation is accomplished by Sirt1 and is essential for the induction of autophagy following nutrient deprivation [90]. This observation correlates with the fact that Sirt1 is implicated in metabolic processes and upregulated during fasting conditions [141]. Stimulation of starvation-induced autophagy requires also hyperacetylation of tubulin, which could explain why the absence of HDAC6 does not prevent this type of autophagy [142].

3.4. Plasma Membrane and Organelles. Several ion pumps of the plasma membrane, like the Na^+/K^+ -ATPase and the Ca^{2+} -ATPase can associate with acetylated microtubules or acetylated tubulin dimers but not with unacetylated tubulin. Association with acetylated tubulin inhibits the pumps and may serve at the same time as an anchorage site for the microtubular network to the plasma membrane [143]. Acetylated tubulin at the plasma membrane is also found in another context. The viral protein gp120 of HIV binds to its receptor CD4 in the plasma membrane of T cells and induces tubulin acetylation and accumulation at contact sites. These plasma membrane domains rich in acetylated tubulin could be entry sites for HIV, since active HDAC6 diminishes HIV infection [144].

Mitochondria harbour an enormous amount of acetylated proteins, and there are several publications on the important role of acetylation for the regulation of metabolic and age-related processes, which could not be included in this paper.

Interestingly, recent studies describe acetylation and deacetylation events occurring in the lumen of the ER and Golgi apparatus, respectively. Bace1, the protease responsible for amyloid precursor protein cleavage, and the LDL receptor become acetylated in the ERGIC [145, 146]. Acetylation is essential for their stabilisation and progression along the secretory pathway. Both proteins are deacetylated in the Golgi apparatus. This implies not only the presence of Hats and HDACs in these cellular compartments but also an ER import system for acetyl-CoA. While the responsible Hat enzymes have been identified and appear to be type II membrane proteins, the HDACs still remain to be discovered and

the acetyl-CoA transporter is currently under investigation [147].

3.5. Nuclear-Cytoplasmic Shuttle. Besides the regulation of protein stability, activity and interactions, acetylation is widely used for regulating the cellular localization of proteins, especially for nuclear import and export (Figure 1(b)). Most proteins, which shuttle between the nucleus and the cytoplasm, are acetylated by p300/CBP, including HNF-4, CIITA, PCNA, SRY, cAbl, CtBP2, p53, PAP, and β -catenin, RECQL4 [148–157]. Two out of those can also be acetylated by PCAF [154, 155], and there is only one report published so far where Gcn5-mediated acetylation plays a role, concerning CDC6 [158]. Until now no general rule for the localisation of the acetylated subpopulation of proteins has been deciphered. For some proteins, acetylation enhances localisation in the cytoplasm [149, 151, 153, 154, 158, 159], whereas for others acetylation will preferentially favour a nuclear localization [148, 150, 152, 155, 156, 160, 161]. The mechanism by which acetylation influences cellular localisation can be either the modification of an interaction with a binding partner leading to retention in a particular compartment, or an altered interaction with nuclear import/export factors. For poly(A) polymerase (PAP) and the DNA helicase RECQL4 it was shown that acetylation disrupts their interaction with the nuclear import factors leading to their accumulation in the cytoplasm [149, 153]. In contrast, SRY acetylation provokes its nuclear import, which also depends on its interaction with a nuclear import factor, but in this case acetylation induces an increased interaction of the acetylated form with importin beta [148]. Interestingly, in order to stay in the nucleus HNF-4 has to be kept in an acetylated state at its NLS. Deacetylation leads to CRM1-dependent export most probably after a conformational change liberating its nuclear export signal [156]. Similarly, cytoplasmic localisation of acetylated p53 depends on CRM1 binding to its nuclear export signal which is accessible only when p53 oligomerization is prevented by lysine acetylation [151]. Another example of CRM1-dependent export regulated by acetylation is NF-kappaB. When present in the nucleus the RelA subunit of NF-kappaB can be acetylated on several lysine residues, which differentially influence its nuclear export mediated by the NF-kappaB inhibitor Ikbalpha [161, 162].

Interestingly, the nuclear import factor importin alpha is itself targeted by reversible acetylation by p300/CBP, which stimulates heterodimer formation with importin beta [163, 164]. In addition, several other transport factors are among the acetylated proteins identified by Choudhary et al. These include importin beta as well as CAS, the exportin responsible for recycling importin alpha to the cytoplasm, and the more general export receptor CRM1 [5].

4. Concluding Remarks

The overwhelming amount of published work on important aspects of cytoplasmic acetylation precludes an exhaustive overview covering the current knowledge (for additional

aspects see [165]). We have therefore focused on selected examples illustrating some important points and wish to apologize for other interesting observations, which have not been included.

Having discussed the importance of acetylation in the cytoplasm, it may be worth insisting on the fact that cells should be considered as entities which cannot be strictly divided into two different compartments. The interpretation of some of the results might have been biased by indirect effects due to transcriptional regulation or leakage of nuclear proteins during biochemical purification steps. Also proteins generally considered as typical cytoplasmic residents do play important roles in the nucleus. Actin, for instance, known as a major cytoskeletal and thus cytoplasmic constituent, also plays important roles in the nucleus as a part of transcription or DNA remodelling complexes. Even tubulin was recently found translocating between the nucleus and the cytoplasm [166]. To circumvent problems related to the presence of the nucleus for the characterisation of cytoplasmic acetylation events, we propose platelets as a good model system. Platelets are devoid of a nucleus but are equipped with all the important players to execute known cytoplasmic processes like exocytosis, adhesion, aggregation, shape change, remodelling of the cytoskeleton, and even apoptosis-related events. Furthermore all these processes have to be tightly regulated and induction has to be extremely rapid to ensure hemostasis and prevent thrombosis.

We would like to conclude with the prediction that, although numerous acetylated proteins have been already identified in the cytoplasm, there are likely more to be discovered. This idea is supported by the fact that acetylation of lysine 40 of alpha tubulin has not been detected in the proteomic survey of acetylated proteins by Choudhary et al. [5] and other acetylation sites may have been missed for the same technical reasons (see above). New strategies including bioinformatic prediction approaches [167, 168] may lead us to new candidates for reversible acetylation. We therefore believe that the story will not end here and future will tell us more about acetylated proteins and exciting new regulatory mechanisms. Finally this paper highlights the importance of reversible cytoplasmic protein acetylation in a wide range of cellular functions and regulatory mechanisms. The functional significance of this modification has been largely recognized in the past for transcriptional control mechanisms in the nucleus and at present, as summarized here, also for cytoplasmic events. Thus the functional role of reversible protein lysine acetylation now definitely approaches the regulatory power of protein phosphorylation as rightly predicted by Kouzarides [169].

Abbreviations

ADP:	Adenosine diphosphate
AhR:	Aryl hydrocarbon Receptor
Arp:	Actin-related protein
ATAC:	Ada Two-A Containing
ATF2:	Activating Transcription Factor 2

ATP:	Adenosine triphosphate
BBIP10:	BBSome Interacting Protein 10
BDNF:	Brain Derived Neurotrophic Factor
Cbl:	Casitas B-lineage lymphoma
CBP:	CREB Binding Protein
CD:	Cluster of Differentiation
CDC6:	Cell Division Cycle 6
CRM1:	Chromosome Region Maintenance 1
EGFR:	Epidermal Growth Factor Receptor
Elp3:	Elongator complex protein 3
ER:	Endoplasmic Reticulum
ERGIC:	Endoplasmic Reticulum—Golgi Intermediate Compartment
Gcn5:	General control of amino acid synthesis 5
Hat:	Histone acetyltransferase
HDAC:	Histone deacetylase
HHV-8:	Human Herpes Virus 8
HIV:	Human Immunodeficiency Virus
HNF-4:	Hepatocyte Nuclear Factor 4
HSP90:	Heat Shock Protein 90
KAT:	lysine (K) acetyltransferase
LDL:	Low-density Lipoprotein
MYST:	MOZ, Ybf2/Sas3, Sas2, Tip60
NAD:	Nicotinamide Adenine Dinucleotide
NF-kappaB:	Nuclear Factor-kappaB
NGF:	Nerve Growth Factor
NLS:	Nuclear Localization Signal
PAP:	Poly A Polymerase
PCAF:	p300/CBP-associated Factor
PCNA:	Proliferating Cell Nuclear Antigen
PDGF:	Platelet-derived Growth Factor
QC-autophagy:	Quality Control-autophagy
Rac1:	Ras-related C3 botulinum toxin substrate 1
RECQL4:	RecQ protein-like 4
Rho:	Ras homolog gene
RNAi:	RNA interference
SAGA:	Spt-Ada-Gcn5 Acetyltransferase
Sirt:	Sirtuin
SRY:	Sex-determining Region Y
STAGA:	SPT3-TAF9-GCN5/PCAF acetylase
TFTC:	TBP-free TAF-containing Complex
Tip60:	Tat interacting protein 60
TNF:	Tumor Necrosis Factor

TPPP:	Tubulin Polymerization-Promoting Protein
tRNA:	transfer RNA.

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Review Article

The Role of HDAC6 in Cancer

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Histone deacetylase 6 (HDAC6), a member of the HDAC family whose major substrate is α -tubulin, has become a target for drug development to treat cancer due to its major contribution in oncogenic cell transformation. Overexpression of HDAC6 correlates with tumorigenesis and improved survival; therefore, HDAC6 may be used as a marker for prognosis. Previous work demonstrated that in multiple myeloma cells, inhibition of HDAC6 results in apoptosis. Furthermore, HDAC6 is required for the activation of heat-shock factor 1 (HSF1), an activator of heat-shock protein encoding genes (HSPs) and CYLD, a cylindromatosis tumor suppressor gene. HDAC6 contributes to cancer metastasis since its upregulation increases cell motility in breast cancer MCF-7 cells and its interaction with cortactin regulates motility. HDAC6 also affects transcription and translation by regulating the heat-shock protein 90 (Hsp90) and stress granules (SGs), respectively. This review will discuss the role of HDAC6 in the pathogenesis and treatment of cancer.

1. Introduction

Inhibition of histone deacetylation has become an accepted target for cancer therapy [1–4]. Cancer, the second leading cause of death in the United States [5], involves the uncontrolled growth of abnormal cells that can spread to other areas of the body [6]. It is generally treated with chemotherapy, such as vincristine, vinblastine, and colchicine bind tubulin and inhibit microtubule (MT) polymerization, which block mitosis. Taxol, on the other hand, stabilizes the MT and blocks cell division [7]. Despite chemotherapy, many patients relapse and die from cancer. The American Cancer Society estimated 565,650 deaths this year [5]; therefore, new targets for cancer therapy need to be investigated.

Histone deacetylases (HDACs) are primarily involved in the deacetylation of histones [8, 9] but some HDACs, such as HDAC6 can also affect the function cytoplasmic nonhistone

proteins. This review will discuss the interaction of HDAC6 with these nonhistone proteins, and discuss how HDAC6 is a key regulator of many signaling pathways that are linked to cancer, thereby making HDAC6 an attractive target.

HDACs are grouped into three classes based on their primary homology to three *Saccharomyces cerevisiae* HDACs [10, 11]. The class I HDACs, which include HDAC1, HDAC2, HDAC3, and HDAC8, are most related to the yeast transcriptional regulator yRPD3. Class II HDACs, which include HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10, share domains similar to yHDA1. HDAC11 is most closely related to class I HDACs; however, no classification has been given since its sequence similarity is too low. Class III HDACs are similar to the NAD⁺-dependent ySIR2 [10, 11].

The deacetylase-trichostatin A (TSA) and deacetylase-suberoylanilide hydroxamic acid (SAHA) crystal structures of a homologue from the bacterium *Aquifex aeolicus* revealed the histone acetylase catalytic core and established a general

mechanism for HDAC inhibition [12]. TSA and SAHA are two types of HDACs inhibitors that have antitumor effects since they can inhibit cell growth and induce terminal differentiation [12]. The conserved catalytic domain of HDAC involves about 390 amino acids, forming a tube-like shape pocket with a wider bottom [12]. Removal of an acetyl group from substrate occurs through a “charge-relay system” consisting of two adjacent histidine residues, two aspartic residues, and one tyrosine residue at the bottom of the pocket [12]. A Zn^{2+} cation binds near the bottom of the pocket and it is coordinated by two additional aspartates and one histidine and also by a water molecule; in general, HDAC inhibitors function by chelating the zinc ion, making the charge-relay system dysfunctional [12].

HDAC6 is a unique member of class II because it contains two homologous catalytic domains [8, 13]. Both catalytic domains of HDAC6 are fully functional HDACs and contribute independently to the overall activity of the HDAC6 protein, (see [8, 14–17] Figure 1). In one study, both HDAC6 catalytic domains were required for full tubulin deacetylase (TDAC) activity [18, 19]; however, in other studies the TDAC activity was attributed to the second domain [20–22]. HDAC6, like all other HDACs, is inhibited by TSA; however, HDAC6 is uniquely resistant to the potent HDAC inhibitors trapoxin-B and sodium butyrate. These drugs were used to show that HDAC6 is a deacetylase for α -tubulin *in vivo*, and *in vitro* [20, 23]. HDAC6 was found not to interact with histones, *in vivo*, however, *in vitro*, HDAC6 is able to deacetylate histones [8]. This result led to the investigation of an HDAC inhibitor that mediates acetylation of proteins other than histones. An HDAC6 inhibitor, known as tubacin (*Tubulin acetylation inducer*), was isolated through a multidimensional chemical genetic screen of 7,392 small molecules and cell-based assay targeting acetylation activity of proteins other than histones [21, 24]. Unlike other histone deacetylase inhibitors, tubacin was found to inhibit the deacetylation of α -tubulin in mammalian cells without affecting the level of histone acetylation, gene-expression, or cell cycle progression [21, 24].

The HDAC6 gene is localized in Xp11.23 (<http://www.ncbi.nlm.nih.gov/>) and was first cloned by two different groups Grozinger et al. [8] and Verdel and Khochbin [13]. It is the largest protein yet identified in humans with 1,216 amino acids [8]. Most HDACs are located in the nucleus; however, class II HDACs, are able to translocate to the cytoplasm [10]. HDAC6 predominates in the cytoplasm due to the NES and the SE14 motifs [10, 14], (Figure 1). HDAC6 is also found on the perinuclear and leading-edge subcellular regions, associated with p150^{glued}-containing motor complex [23]. It contains a unique ubiquitin-binding zinc-finger domain (ZnF-UBP domain, also known as the PAZ, BUZ or DAUP domain) in its C-terminal region [25] and a dynein-binding domain (DBD) [7] (Figure 1). Other HDACs such as HDAC10 or a spliced variant of HDAC9 can be localized both in the nucleus and cytoplasm, and HDAC11, which localizes in the nucleus, has been reported to coprecipitate with HDAC6 in the cytoplasm [10]. The expression levels of HDAC6 were observed in the heart, liver, kidney, testis, brain, and, pancreas [8, 13].

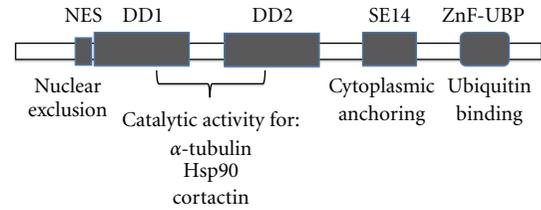


FIGURE 1: HDAC6 functional domains. The only HDAC with two tandem catalytic domains, deacetylase domains (DD1 and DD2). Tubulin, Hsp90, and cortactin have been found to be HDAC6 substrates. The nuclear export signal (NES) prevents accumulation of the protein in the nucleus and the Ser-Glu-containing tetrapeptide (SE14) region ensures a stable anchorage of the enzyme in the cytoplasm. The linker between both DDs is able to bind dynein and the high affinity ubiquitin-binding zinc finger domain (ZnF-UBP).

2. HDAC6 Expression and Cancer

Expression profiling analysis, with cDNA microarray in MCF-7 cells, showed that the HDAC6 gene is an estrogen-regulated gene [26]. Estrogens play an important role in the normal growth of mammary glands, as well as in the development of estrogen-dependent breast cancer [27]. The estrogen receptor α (ER α) status of breast cancer patients is widely used as an indicator for endocrine therapy responsiveness and for prognosis, but 30–40% of the ER α positive patients do not respond to the therapy, and 10% who are ER α negative do respond [3, 27].

The estrogen-mediated up-regulation of HDAC6 suggests that there might be a link between the levels of HDAC6 expression and metastasis of breast cancer that could be useful in the prognosis of patients. One study involving quantitative real-time reverse transcription-PCR, as well as immunohistochemistry in 135 female patients with invasive breast cancer showed that higher levels of expression of HDAC6 mRNA correlated with tumor size (less than 2 cm), low histologic grade, and ER α and progesterone receptor-positive tumors [27]. The high level of HDAC6 mRNA expression also correlated with better prognosis in terms of disease-free survival and better response to endocrine treatment. In a different study, the expression levels of HDAC6 were evaluated by immunohistochemical staining of 139 consecutively archived human breast cancer tissues [3]. In this study, a subset group of ER-positive patients with higher levels of HDAC6 expression who received adjuvant treatment with tamoxifen (TAM), a selective estrogen receptor modulator, resulted in improved survival. These results suggest that HDAC6 could potentially serve as an additional marker for prognosis in breast cancer patients.

The overexpression of HDAC6 has been identified in a variety of other cancer cell lines and mouse tumor models. The expression levels of HDAC6 in ovarian cancer cells and tissues were higher in low-grade and high-grade ovarian carcinomas compared with benign lesions and immortalized ovarian surface epithelium cell lines [28]. HDAC6 expression was also investigated in retrovirus expressing SV40 early region or/and Ras^{G12V} transformed and nontransformed

human embryonic kidney cells (HEK), prostate epithelial cells (PrEC), and mouse embryonic fibroblast (MEF) cells and it was found that oncogenic Ras can lead to up-regulation of HDAC6 [29]. HDAC6 expression was also up-regulated in primary oral squamous cell lines and its level of expression correlated with primary tumor stage [30]. In a different study, HDAC6 was consistently overexpressed in primary acute myeloid leukemia (AML) blasts and in some myeloblastic cell lines [31]. The up-regulation of HDAC6 in diverse tumors and cell lines suggests an important role in cancer and it is therefore being widely investigated.

3. HDAC6 Is Required for Oncogenic Cell Transformation

A study performed by Lee et al. [29] found that HDAC6 is required for efficient oncogenic tumorigenesis by providing anchorage-independent proliferation to transduced cells. MEFs derived from wild type HDAC6-null embryos and transduced with retrovirus expressing SV40 early region and Ras^{G12V} resulted in >10-fold fewer colonies in the HDAC6-null oncogenic transduced MEFs samples than in the wild type oncogenic transduced MEFs. These results strongly indicated that HDAC6 is required for Ras-induced oncogenic transformation by providing anchorage-independent proliferation, a hallmark of malignant transformation. Anchorage-independent proliferation allows the cells to survive by escaping anoikis, a type of cell death due to lack of sufficient cell adhesion to the surrounding basement membrane and extracellular matrix (ECM) [29].

HDAC6 is required to maintain the transformed phenotypes of a number of established oncogenic cell lines and for tumor growth. Knockdown of HDAC6 in SKOV3 ovarian cancer, SKBR3 breast carcinoma, and MCF7 breast cancer cell lines inhibited the anchorage-independent growth of those cancer cells by 5-fold to 30-fold [29]. This suggests that HDAC6 plays a major role in the survival of tumor cells. To test whether HDAC6 was critical for tumorigenesis, immunocompromised severe combined immunodeficient (SCID)-Beige mice were subcutaneously injected with either HDAC-6 specific shRNA or scrambled control and it was found that the growth of HDAC6 knockdown cells was significantly retarded [29]. Furthermore, HDAC6 knockdown cells reconstituted with wild type HDAC6, but not with catalytically inactive mutant HDAC6, regained its phenotype, indicating that HDAC6 is specifically required for tumorigenic growth. Further studies of the role of HDAC6 and tumorigenesis were performed using 7,12-dimethylbenzanthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) carcinogen-induced spontaneous tumorigenesis [29]. After tumor induction, the tumors decreased by half and the formation of tumors was retarded by two weeks in the HDAC6 null mice. These results suggested that HDAC6 is required for optimal tumor growth. RAS/MAPK signaling pathway is known to be required for tumorigenesis, and this study also showed that HDAC6 plays a major role in this pathway. HDAC6 knockout mice demonstrated reduced phosphorylation of

AKT and ERK1/2, signaling pathways involved in tumor growth, and lower levels of activated Ras than those derived from wild-type mice. Thus, HDAC6 is considered to be required for the efficient activation of the oncogenic Ras signaling pathway. These results indicate that HDAC6 is a major contributor of tumorigenesis and maintenance of the transformed phenotype.

4. HDAC6 Inhibition and CYLD Activation Lead to a Delay in the Cell Cycle

In a different study of HDAC6 and its role in tumorigenesis, it was found that CYLD-mediated inhibition of HDAC6 was essential for CYLD activation and delay in the cell cycle. CYLD is a tumor-suppressor gene present in patients suffering from cylindromatosis, a benign skin tumor [32]. In CYLD-transduced melanoma cells, as well as other tumor cell lines, CYLD constitutively colocalizes with acetylated MTs in the perinuclear region, where as in primary mouse keratinocytes melanoma cells or primary human melanocytes, CYLD perinuclear localization occurs after TPA treatment or exposure to UV light [30]. In this study, it was found that keratinocytes and melanoma cells that were treated with TSA or tubacin yielded increased levels of acetylated tubulin in enhanced green fluorescent protein (EGFP)-tagged expressing melanoma cells, while treatment of EGFP-CYLD-expressing melanoma cells with TSA or tubacin failed to increase acetylated α -tubulin. These results indicated that CYLD endogenously induces acetylation of α -tubulin by inhibiting HDAC6. Furthermore, it was found that inhibition of HDAC6 by the N-terminal cytoskeleton-associated protein-glycine-conserved (CAP-GLY) domains of CYLD mediated inhibition of HDAC6 [32].

CYLD-mediated HDAC6 inhibition was observed to enhance its association with the MTs, reduce the rate of cytokinesis, and delay cell cycle (Figure 2). Inhibition of BCL-3, a proto-oncogene, by the deubiquitinase activity of CYLD is mediated by an additional signal induced by TPA and requires intact acetylated MTs [32]. CYLD-mediated inhibition of HDAC6 results in perinuclear translocation of CYLD, and this in turn mediates BCL-3 inhibition by blocking its nuclear translocation. The lack of BCL-3 in the nucleus inhibits the transcriptional activity of NF- κ B p50/p52, reduces the expression of cyclin-D1, and delays G₁/S transition of the cell cycle [32] (Figure 2). These studies demonstrated how inhibition of HDAC6 can prevent tumor growth, indicating that HDAC6 should be considered as a major target in transformed cells.

5. The Role of HDAC6 during Cellular Stress Response and Tumorigenesis

A defense mechanism known to counteract the toxic effects of misfolded protein accumulation is carried out by the proteasome and the aggresome pathway; this in turn gives a survival advantage to transformed cells. A purified HDAC6-containing complex from mouse testis cytosolic extracts revealed the presence of vasolin-containing

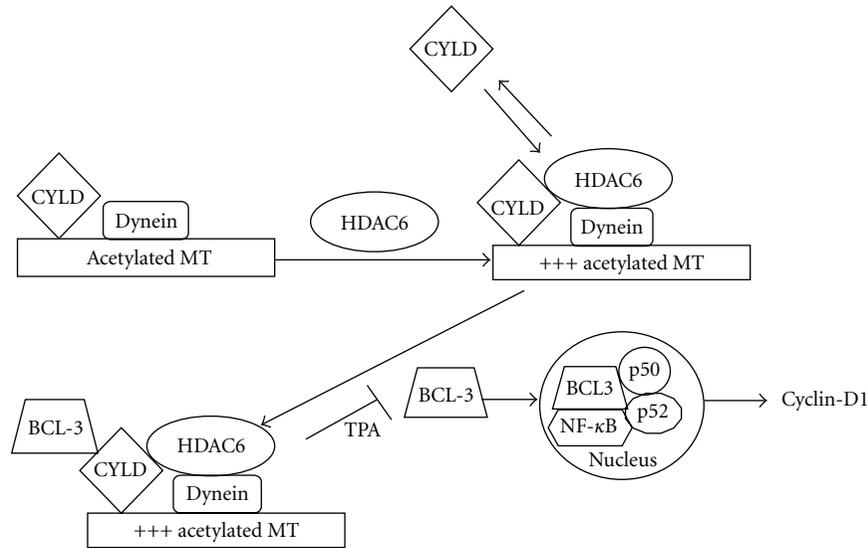


FIGURE 2: CYLD-mediated inhibition of HDAC6 leads to a delay in the cell cycle. CYLD binds acetylated microtubules and inhibits HDAC6. Inhibition of HDAC6 leads to increased levels of acetylated α -tubulin and this induces more binding of CYLD to MTs. CYLD binds BCL-3, and in the presence of TPA blocks its nuclear translocation, thereby inhibiting cyclin-D1.

protein/protein 97 (VCP/p97) and phospholipase A2-activating protein/ubiquitin fusion degradation protein 3 (PLAP/UFD3), two proteins that are known to control protein ubiquitination [25]. HDAC6 contains a ZnF-UBP domain and a dynein-binding domain (Figure 1). These findings led to the hypothesis that HDAC6 participates in cargo sorting and transport by regulating the acetylation of MTs and by binding ubiquitinated proteins; therefore, the effect of HDAC6 on MTs and its role in protein degradation in response to cell stress are widely being investigated.

6. Microtubule Stabilization Leads to Increased Levels of HDAC6-Mediated Acetylated α -Tubulin

MTs play a role in cell cycle division (mitotic spindle), intracellular protein trafficking, shape maintenance, cell motility and attachment, and in signal transduction [44], hence, they are validated targets for anticancer therapy [45]. MTs are components of the cytoskeleton and they are composed of the tubulin molecule, $\alpha\beta$ tubulin dimer (Mr \sim 100,000), which polymerizes to form 13 linear protofilaments with a rigid hollow rod of approximately 25 nm in diameter and several microns in length [7, 46]. The MTs also bind proteins that are collectively known as the MT associated proteins (MAPs). In animal cells, the major MT organizing center (MTOC) is the centrosome [7, 46]. The MTs undergo rapid cycles of assembly and disassembly and it has been established that their instability arises from the hydrolysis of GTP bound to β -tubulin during or shortly after polymerization [7]. Cytoplasmic MTs, which polymerize and depolymerize at high rates, in the presence of taxol, an antimitotic drug, bind the drug and are stabilized. This MT assembly allows for acetylation of α -tubulin to occur

[20, 23, 47]. Therefore, the reversible acetylation on the ϵ -amino group of Lys40 near the N-terminus of α -tubulin is coupled to MT turnover [47].

HDAC6 plays a role in regulating the stability of dynamic MTs [18, 20, 23]. It was observed that *in vivo*, depolymerized tubulin is rapidly deacetylated while tubulin acetylation generally occurs on stable or polymerized MTs [18, 20, 23]. Furthermore, a group of researchers reported that combination of farnesyl transferase inhibitor lonafarnib and taxol enhance HDAC6-dependent tubulin deacetylation in breast and cell lung carcinoma cells [2].

7. HDAC6 and its Role in the Aggresome Pathway in Response to Cellular Stress

Targeting the proteasome or the aggresome to induce apoptosis or programmed cell death is one of the methods to target cancer cells. Transformed cells accumulate more misfolded proteins, which are disposed of by the proteasome and the aggresome [33, 48, 49]; therefore, cancer cells are more dependent on the proteasome and aggresome activity than nonmalignant cells for cell survival [33]. Upon inhibition of the proteasome, the high binding affinity of HDAC6 to ubiquitinated proteins activates the aggresome pathway [14] (Figure 3). Misfolded proteins form particulate (\sim 200 nm) miniaggregates [36]. HDAC6 binds the ubiquitinated proteins and delivers them to the motor protein dynein, which in turn, transports the cargo along the MTs to the MTOC, where intermediate filament cytoskeleton surrounds the particle to form an aggresome [33, 36]. The aggresome is dynamic and it recruits chaperones and proteasomes [36]. Once this occurs, it activates the autophagic clearance and the autophagosomes fuse to lysosomes resulting in the degradation of the misfolded proteins [50]. The HDAC6

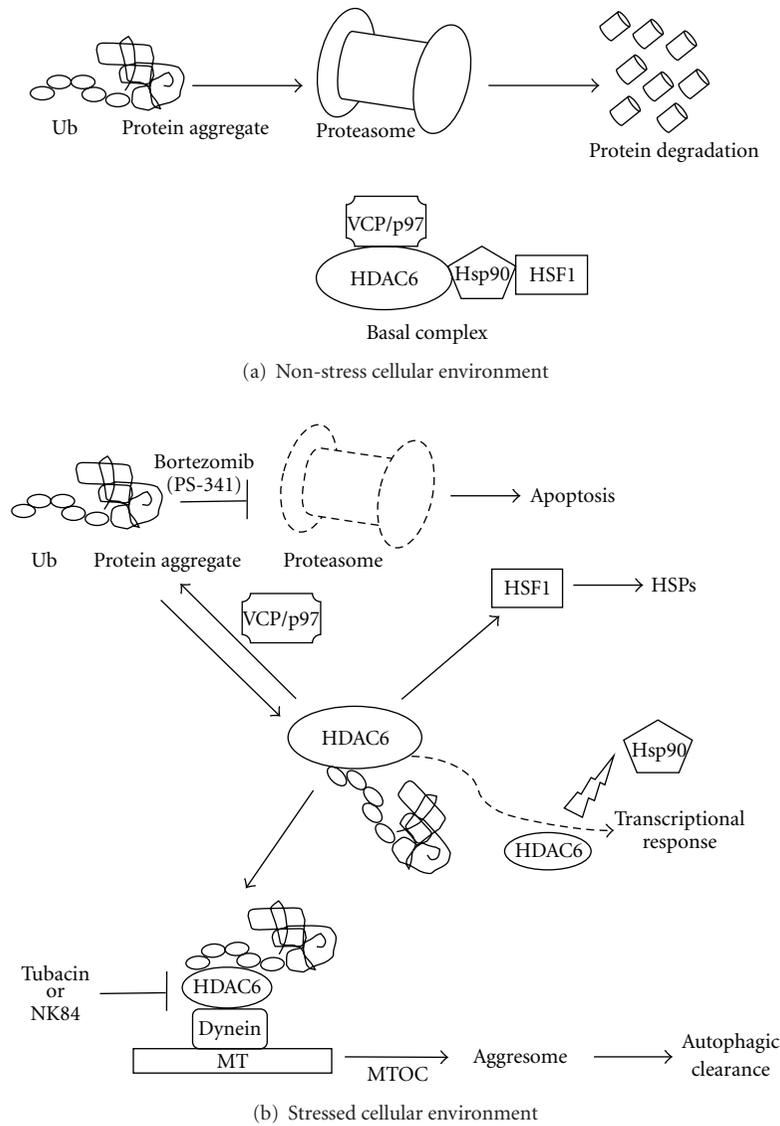


FIGURE 3: Cell stress response: activation of the aggresome pathway, HSPs, and transcriptional response. (a) Under nonstress conditions, protein degradation occurs through the proteasome. HDAC6 is sequestered by VCP/p97 and forms a basal complex with HSF1 and HSP90. This keeps HSF1 in its inactive form. (b) Inhibition of the proteasome leads to an increase of misfolded proteins. HDAC6 binds these ubiquitinated protein aggregates and the basal complex disassembles. This complex dissociation activates HSF1, which eventually activates HSPs. The protein aggregates are transported along the MTs to MTOC to form an aggresome and autophagic clearance is activated. HDAC6 can also modulate Hsp90 acetylation levels and affect the maturation of client proteins, such as the glucocorticoid and androgen receptors, impairing gene transcription activation.

partner VCP/p97 is a chaperone with segregase activity. It disassembles the ubiquitinated aggregate/HDAC6 complex by extracting or sequestering HDAC6, so that the ubiquitinated proteins can be degraded through the proteasome instead of the aggresome [14, 36].

To study the effects of HDAC6 inhibition on the stress response, the HDAC6 inhibitor, tubacin, was used to test its effect on multiple myeloma cells. Tubacin-mediated α -tubulin acetylation disrupted HDAC6-dynein complex and synergistically increased the bortezomib-induced apoptotic effect in myeloma cells by c-Jun NH₂-terminal kinase/caspase activation [37]. Multiple myeloma is cancer

of plasma cells and can become resistant to conventional chemotherapy. The combination of bortezomib and tubacin was able to inhibit the proliferation of myeloma cells *in vitro*. [37]. More importantly, it was found that tubacin did not have an effect on normal peripheral blood mononuclear cells [37], hence the inhibition of HDAC6 by tubacin may be used for combined chemotherapy to treat myeloma or other types of cancer.

In ovarian cancer cells, their elevated metabolic activity results in increased ubiquitin proteasome-system (UPS) stress and this in turn, leads to an up-regulation of HDAC6 expression [28]. In one study, ovarian cancer cells were

treated with HDAC6-specific inhibitors, tubacin and its derivative NK84. The treatment significantly inhibited the growth/survival of the ovarian cancer cell lines relative to the immortalized ovarian surface epithelial (IOSE) cell lines. Combined inhibition of both the proteasome and HDAC6-mediated aggresome degradation pathways is targeted with PS-341 and NK84, respectively, and the results showed synergistic cell death induced by both drugs. Furthermore, immunofluorescence analysis of the polyubiquitinated proteins in ovarian cancer cells, treated with both PS-341 and NK84 drugs, showed that HDAC6 inhibition prevented aggresome formation [28].

8. HDAC6 Is Required for Activation of HSF1 and Heat-Shock Protein Encoding Genes Response

An elegant study performed by Boyault et al. [34] using primary cells and established cell lines isolated from HDAC6-deficient mice, or human HDAC-6 deficient cells re-expressing wild type or mutated HDAC6, found that VCP/p97 is recruited by HDAC6 to a heat-shock protein 90/heat-shock factor 1 (Hsp90/HSF1) complex to form a “basal complex” in unstressed cells (Figure 3). At baseline, Hsp90 maintains HSF1 in its inactive form. HSF1 is a transcription factor that is essential in the activation of HSPs in response to the accumulation of misfolded proteins caused by heat shock or proteasome inhibition [29, 34] (Figure 3). The HSP response helps to reduce protein aggregate toxicity by increasing the expression of major cellular chaperones that either disaggregate proteins and help in their normal refolding or deliver them to the proteasome [35, 51]. Upon proteasome inhibition, HDAC6 binds the ubiquitinated aggregates and dissociation of HDAC6 and Hsp90 from HSF1 occurs. In the absence of VCP/p97, the Hsp90/HSF1 complex remains; therefore, the VCP/p97 ATPase activity seems to play a role in the dissociation of the Hsp90/HSF1 complex [34].

The essential role of HDAC6 in the activation of HSF1 is in accordance with the findings that similar to HDAC6-deficient cells and HDAC6-null mice, HSF-1-null MEFs were found to suppress skin tumor formation induced by DMBA-TPA treatment and oncogenic Ras-mediated anchorage-independent proliferation [29, 35]. Both HSF1 and HDAC6 regulate the oncogenic RAS/MAPK signaling pathway, and their presence is required for maintenance of transformed phenotypes in established tumor cells [29, 35].

9. HDAC6-Mediated Transportation of Stress Granules Leads to Reversible Translational Suppression in Response to Cellular Stress

HDAC6 also plays an essential role in a different cellular response induced by environmental stress. Kwon et al. [43] found that either of the two HDAC6 domains is sufficient to interact with cytoplasmic stress granule (SG) components, which are involved in reversible translational suppression in response to cellular environmental stress. The deacetylase

activity and the ubiquitin-binding function of HDAC6 were found to be essential in the formation of SGs [43]. HDAC6 recruits the SGs to motor protein via binding of another SG protein, Ras-GTPase-activating protein SH3 domain-binding protein 1 (G3BP1) to allow SGs movement along the MTs. SGs are also ubiquitin positive and this allows direct binding to HDAC6 and MTs. In this study, disruption of MTs or motor proteins was found to inhibit individual SG components along the MTs. This type of HDAC6-mediated stress response strongly suggested a role of HDAC6 in the control of RNA metabolism and translation. It is worth mentioning that in a different study using cultured HSF-1 deficient MEFs under growth factor-depleted conditions or serum starvation, reduced levels of three ribosomal subunits, L26RP, L28RP, and S6RP, as well as those of phosphorylated ribosomal protein S6 kinase (p70 S6K), were observed [35]. p70 S6K is known to be a regulator of translational activity [35]. These results suggest that HSF1, which is activated by HDAC6, plays a role in ribosome biogenesis and protein translation, pathways that contribute to cancer [35].

10. HDAC6 Modulates Cell Motility

Cell motility plays a key role in tumor metastasis; hence, fibroblast motility has been used as a model to study the mechanisms of tumor cell migration *in vivo* [52]. Motile fibroblast contains two different types of MTs: The highly dynamic MTs predominate randomly throughout the cell periphery, and the less dynamic ones are found between the fibroblast nucleus and its leading edge [53]. In fibroblast crawling, actin is a key player, however, the MTs also play important roles, since MTs are required for the establishment and maintenance of cell polarity and directional movement [52]. Fibroblasts were observed to require dynamic MTs for the remodeling and turnover of their focal adhesions [54]. The detachment from the extracellular matrix can slow down invasion migration, so adhesion turnover is rate-limiting for rapid migration of fibroblasts [55]. Nevertheless, dependence for cell polarity on MT dynamism is seen only in some cell types; for example, fibroblasts and epithelial cells, which require an intact MT cytoskeleton to migrate during wound healing [56]. Faster migrating cells, such as leukocytes, do not require an intact MT cell cytoskeleton [56].

Studies of cell motility revealed that overexpression of HDAC6 increases chemotactic cell motility, suggesting that deacetylation of at least one cytoplasmic HDAC protein enhances motility [23]. HDAC inhibitors, such as TSA and SAHA block invasive cell motility by altering gene expression as a result of hyperacetylated HDAC nuclear substrates, such as histones or transcription factors [38]. Studies have shown that, as expected, tubacin leads to a reduction in cell motility [21, 38]. The inhibition of HDAC6 by tubacin, which leads to the hyperacetylation of tubulin, blocks fibroblast invasion motility similarly to the inhibition of all HDACs by TSA [38]. It was observed that in HDAC6-inhibited cells, MT dynamics is decreased, leading to an increase of focal adhesion accumulation, thus a decrease in fibroblast motility

TABLE 1: Overview of HDAC and its role in cancer.

Tumorigenesis	HDAC6 expression and its mediated HSF1 activation are essential for tumor growth and maintenance of oncogenic phenotype by promoting anchorage-independent proliferation to transformed cells [3, 26–35]. CYLD-mediated HDAC6 inhibition leads to delay in the cell cycle and reduced rate of cytokinesis [30, 32].
Cell survival	HDAC6 binds ubiquitinated protein aggregates and this leads to the dissociation of the basal complex, and eventual activation of the aggresome pathway [28, 33, 36]. HDAC6 inhibition leads to apoptosis [37].
Cell motility or metastasis	HDAC6 overexpression leads to increased cell motility [3, 21, 28, 38]. HDAC6 inhibition leads to hyperacetylated cortactin and impaired cell motility [15, 17]. HDAC6 inhibition also leads to its impaired catalytic domain affecting MT dynamism [39].
Transcriptional response	HDAC6 deacetylates Hsp90 and prevents the maturation of glucocorticoid and androgen receptors [40–42].
Translational response	HDAC6 forms a complex with SGs and G3BP1 and eventually induces a reversible translational suppression [43].

[38]. Another group performed a scratch assay and trans-Matrigel migration assays and showed that NK84-mediated inhibition of HDAC6, in ovarian cancer cell lines retarded spreading and inhibited migration, respectively [28].

In a different study, treatment of ER α positive breast cancer MCF-7 cells with estradiol or stably transfected with HDAC6 revealed that HDAC6 is a novel estrogen up-regulated gene and its expression led to increased cell motility [3]. Saji et al. [3] also found that either TAM, the pure antiestrogen ICI 182,780, or tubacin, prevented the estradiol-induced HDAC6 accumulation and deacetylation of α -tubulin, leading to reduced motility.

HDAC6 can also interact with a different substrate, cortactin, *in vivo* and *in vitro*, and both HDAC6 catalytic domains are necessary for interaction [17], (Figure 1). Cortactin is an acetylated protein found at areas of dynamic actin assembly, such as the leading edge of migrating cells [17]. This protein is originally known as a substrate of Src tyrosine kinase and it plays a role in regulating cell motility [57, 58]. Cortactin has been found to be overexpressed in several carcinomas [58]. It interacts with F-actin to promote polymerization and branching by modulating a “charge patch” in the cortactin repeat region [15]. Zhang et al. [15] found that inhibition of HDAC6 leads to hyperacetylation of cortactin and prevents its translocation to the cell periphery, blocks association with F-actin, and impairs cell motility.

In a different study, HDAC6 was found to play an important role in the chemotaxis of T-lymphocytes, independent of its deacetylase activity. The knockdown of HDAC6 expression in T-cells decreased their chemotactic capability; however, HDAC6 inhibitors, such as TSA and tubacin, showed that the deacetylase activity of HDAC6 was not involved in the modulatory effect of HDAC6 on cell migration [59]. Cabrero et al. [59] suggested that HDAC6 might act as an on/off switch, regulating the rapid formation (10 to 20 folds faster than fibroblasts) of molecular complexes required for lymphocyte migration. In favor of that hypothesis the results of two studies were mentioned. First, HDAC6 reversibly complexes with phosphatases such as protein phosphatase 1 (PP1) and upon HDAC inhibition, release of PP1 from its complex with HDAC6 leads to the dephosphorylation of Akt, which in turn affects cell

migration. The second finding was that tubulin acetylation decreases with inhibition of RhoA activity and since Rho GTPases coordinate both poles of migrating T-lymphocytes and control the dynamics of actin and tubulin cytoskeleton, HDAC6 may act as a mediator between actin- and tubulin-associated proteins to regulate motility [59].

A recent study involving the effect of HDAC6 on MT found that the impaired catalytic domain of HDAC6 is responsible for MT dynamics. In two different studies using NIH 3T3 fibroblast or A549 cells, it was found that MT stabilization is not promoted by tubulin acetylation [21, 60]. Palazzo et al. [60] reported that reduced motility is a consequence of alterations in the degree of tubulin acetylation and not of MT stabilization. Zilberman et al. [39] showed that the impaired catalytic domain of HDAC6, induced by HDAC6 inhibitors, TSA or tubacin, or point mutations in either of the two catalytic domains, is responsible for the regulation of MT dynamics, rather than tubulin acetylation alone. In this study, HDAC6 knockdown increased tubulin acetylation, but had no effect on the MT growth velocity, in the presence or absence of tubacin.

11. HDAC6 Modulates the Acetylation Status of Hsp90 and Represses Transcription

HDAC6 was also shown to have a third substrate, heat shock protein 90 (Hsp90), and affect transcriptional activation (Figure 3). Hsp90 functions by facilitating the structural maturation and complex formation of client proteins, including steroid hormone receptors and selected kinases [40, 41]. HDAC6 was found to regulate the activity of Hsp90. Inactivation or knockdown of HDAC6 led to Hsp90 hyperacetylation, its dissociation from cochaperone p23, and loss of chaperone activity. The loss of Hsp90 activity prevents the maturation of the glucocorticoid receptor (GR), affecting ligand binding, nuclear translocation, and transcriptional activation [40, 42]. In a different study, knockdown of HDAC6 in prostate cancer (PCa) C4-2 cells led to the hyperacetylation of Hsp90, impaired ligand-independent nuclear localization of endogenous androgen receptor (AR), inhibited prostate-specific antigen (PSA) expression, and cell

growth in the absence or presence of dihydrotestosterone (DHT) [41]. These findings suggest that HDAC6 potentially affects the activity of other client proteins by regulating Hsp90. Nevertheless, other studies have shown that HDAC6 is related to cell migration rather than to transcriptional regulation [21, 23].

12. Conclusions

HDAC6 should be considered a target for cancer therapy due to its essential role in many signaling pathways that provide an advantage to malignant cells to survive and maintain its phenotype (Table 1). HDAC6 as well as its substrate HSF1 are essential for efficient oncogenic transformation by supporting anchorage-independent proliferation to prevent anoikis. HDAC6 and HSF1 can also regulate oncogenic Ras/MAPK signaling pathways, which are required for efficient tumor growth. High levels of HDAC6 expression were found to correlate with tumorigenesis, and ER α positive breast cancer patients who received adjuvant treatment with TAM showed better survival prognosis. Thus, HDAC6 could potentially be used as an additional marker for prognosis. HDAC6 is required for the activation of HSF1, a master activator of HSPs, leading to massive expression of chaperones; thereby, contributing to oncogenic transformed cell survival. HDAC6 is involved in the activation of SGs leading to reversible translational suppression, thus, helping transformed cells survive cellular environmental stress. HDAC6 can also regulate other proteins such as Hsp90, leading to the inhibition of steroid receptors activity and transcriptional activation.

Studies of HDAC6 and its role in cell motility suggest its participation in cancer metastases and migration. The impaired catalytic domain of HDAC6 was found to be responsible for the regulation of MT dynamics. Recent studies have also shown that the inhibition of HDAC6 by tubacin leads to the accumulation of misfolded ubiquitinated proteins and apoptosis in multiple myeloma cells. The effects of HDAC6 on protein degradation and apoptosis revealed that this protein participates in cargo sorting and transport by regulating the acetylation of MTs and binding to ubiquitinated proteins. It is possible that the same HDAC6-mediated protective effect in response to cellular stress is observed in other cells; therefore, more studies using different cancer cell lines should be performed. Furthermore, since inhibition of HDAC6 stabilizes the MTs, other potential apoptotic pathways that inhibit oncogenic tumorigenesis need to be investigated.

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Review Article

Physiological Roles of Class I HDAC Complex and Histone Demethylase

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Epigenetic gene silencing is one of the fundamental mechanisms for ensuring proper gene expression patterns during cellular differentiation and development. Histone deacetylases (HDACs) are evolutionally conserved enzymes that remove acetyl modifications from histones and play a central role in epigenetic gene silencing. In cells, HDAC forms a multiprotein complex (HDAC complex) in which the associated proteins are believed to help HDAC carry out its cellular functions. Though each HDAC complex contains distinct components, the presence of isoforms for some of the components expands the variety of complexes and the diversity of their cellular roles. Recent studies have also revealed a functional link between HDAC complexes and specific histone demethylases. In this paper, we summarize the distinct and cooperative roles of four class I HDAC complexes, Sin3, NuRD, CoREST, and NCoR/SMRT, with respect to their component diversity and their relationship with specific histone demethylases.

1. Introduction

Eukaryotic chromosomes consist of two cytologically defined structures, euchromatin, and heterochromatin. Euchromatin contains transcribable genes, which are subject to either activation or inactivation depending on the cellular situation, whereas heterochromatin contains transcriptionally inert genes. Structural changes in chromatin are tightly linked with posttranscriptional modifications of the histone tails. The combinations of histone modifications establish local and global patterns of chromatin structure and define specific downstream events [1]. These patterns are highly dynamic and can be altered by multiple extracellular and intracellular stimuli. Thus, chromatin has been proposed to serve as a signaling platform by which various signaling pathways are integrated.

Among the numerous covalent modifications identified so far, acetylation and methylation play central roles in chromatin dynamics [2]. Histone acetylation on lysine residues is primarily associated with gene activation, and its levels are controlled by two counteracting enzymes: histone acetyltransferases (HATs) and histone deacetylases

(HDACs). Many lysine and arginine residues on histones are target sites of methylation. In contrast to acetylation, histone methylation is linked with both gene activation and inactivation and is regulated by histone methyltransferases (HMTs) and histone demethylases (HDMs). In most cases, these histone-modifying enzymes are present in multisubunit protein complexes, in which the other components are thought to regulate enzyme activities, modulate substrate recognition, recruit other cofactors, or carry out other undefined functions.

In metazoans, HDACs are divided into three classes, Class I to Class III, based on their sequence similarity. Class I HDACs, which include HDAC1, HDAC2, and HDAC3, are found in four distinct multiprotein complexes, the Sin3, NuRD, CoREST, and NCoR/SMRT complexes. These complexes are highly conserved and function in distinct cellular processes including cell-cycle regulation [3, 4], maintenance of stem cell pluripotency [5], self-renewal, and cellular differentiation. Recent studies have revealed that all of these complexes are associated with specific HDMs. The HDMs are classified into two distinct enzyme families: the nuclear amine oxidase homologs and the JmjC-domain

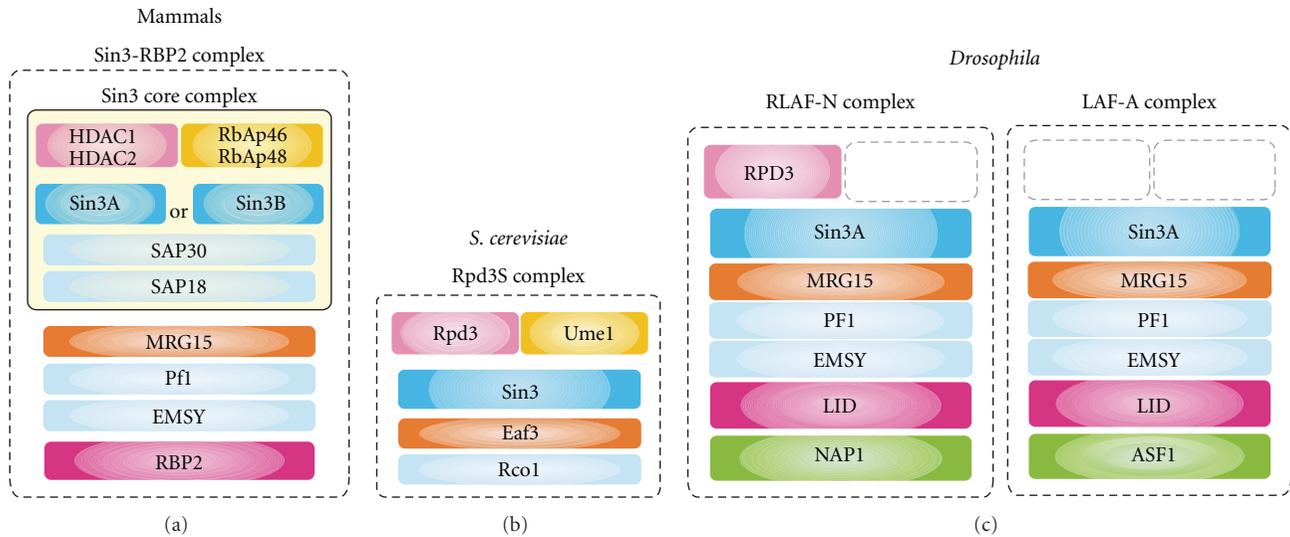


FIGURE 1: *The Sin3 complexes.* Schematic representation of the Sin3-containing HDAC complexes: (a) the Sin3-core and Sin3-RBP2 complexes in mammalian cells; (b) the Rpd3S complex in *S. cerevisiae*; (c) RLAf-N and LAF-A complexes in *Drosophila*. The empty dotted boxes indicate missing subunits compared with the Sin3-core complex.

proteins. LSD1 (KDM1A, also known as AOF2/BHC110) is a member of the former family and was the first histone lysine demethylase identified; it preferentially demethylates methylated histone H3 lysine 4 (H3K4me) [6]. The latter enzyme family, the JmjC-domain proteins, plays important roles in development and cellular differentiation [7–10]. This family has 27 members in mammals, and these members are divided into 11 subgroups based on similarity within the JmjC domain [11]. So far, seven of these subgroups have been shown to have demethylase activity for specific residues on histone H3 or H4.

To achieve strict transcriptional regulation in the complicated chromosomal architecture of the nucleus, it makes sense that eukaryotic organisms developed a system involving combinatorial histone modifications and protein complexes that contain multiple enzymatic activities, such as HDAC and HDM. It is noteworthy that cells produce a variety of HDAC-containing complexes by replacing an integral component with one of its subtypes. These subtype-specific HDAC complexes appear to play distinct roles in particular cellular contexts. In addition, incorporating sequence-specific DNA-binding proteins, such as zinc-finger proteins, can also modulate the function of the HDAC complex. In this paper, we focus on how HDAC-HDM interactions and subtype-specificity regulate functions of the Class I HDAC-containing complexes, in transcriptional regulation and other cellular processes.

2. The Sin3 Complex

The HDAC-containing multiprotein complex, Sin3 complex, is highly conserved from yeast to humans. In mammalian cells, the Sin3 complex consists of six core subunits, HDAC1/2, RbAp46, RbAp48, Sin3A/Sin3B, SAP18, and

SAP30 (Figure 1(a)) [12–14]. Mammalian Sin3A and Sin3B (mSin3A and mSin3B) show high sequence similarity and an overlapping expression pattern, and both are essential in mouse development. However, these subtypes play distinct and nonoverlapping roles. mSin3A has a critical role in the early developmental preimplantation stage, since no mSin3A-null zygotes are found at E6.5 [15]. mSin3A-depleted mouse embryonic fibroblasts (MEFs) exhibit the deregulation of genes involved in cell-cycle control, DNA replication, DNA repair, apoptosis, chromatin modification, and mitochondrial metabolism. Transcriptome analysis has revealed that mSin3A modulates the transcriptional network controlled by Myc-Mad, E2F, or p53. On the other hand, mSin3B has an essential function at the late-gestation stage, and its mutant is different from the mSin3A^{-/-} embryo [16]. In particular, erythrocyte and granulocyte differentiation and G0/G1 cell-cycle control are impaired in the mSin3B^{-/-} embryo, and these phenotypes are due to the derepression of E2F target genes.

Transcriptional regulation is tightly coupled with dynamic change of histone acetylation in the promoter regions. However, the Sin3 complex functions not only at promoter regions but also at transcribed regions. In *Saccharomyces cerevisiae*, Sin3 and a Class I HDAC, Rpd3, are involved with two functionally distinct complexes, Rpd3L and Rpd3S [17]. Both complexes contain Sin3, Rpd3, and Ume1. In addition to these core subunits, Rpd3L contains at least six unique components: Rxt1, Rxt2, Dep1, Sds3, Pho23, and Sap30, whereas Rpd3S contains Rco1 and Eaf3 as unique components (Figure 1(b)) [18]. Rpd3L is localized primarily to promoter regions. In contrast, Rpd3S is localized to transcribed regions that are enriched in methylated H3K36 (H3K36me). H3K36me is a mark catalyzed by Set2 (KMT3) and is tightly coupled with the transcriptional elongation processes [19]. Rpd3S recognizes this H3K36me mark via

a combined action of chromodomain of Eaf3 and PHD (plant homeobox domain) of Rco1 [20–22]. Deletion of Set2 or one of the Rpd3S-specific components results in spurious transcripts that emerge from incorrect transcription start sites in some transcriptionally active genes [17]. Since the H4 acetylation levels within transcribed regions are increased in these mutant cells, Rpd3S is thought to repress unfavorable transcription by maintaining transcribed regions in a hypoacetylated state. In metazoans, however, the relationship between mSin3 complexes and the emergence of aberrant transcripts remains unclear.

One of the Rpd3S-specific components, Eaf3, is highly conserved from yeast to humans. The human Eaf3 homologue MRG15 was initially identified as a factor closely related to MORF4 (mortality factor on human chromosome 4), whose transient expression induces senescence in a subset of human tumor cell lines [23, 24]. MRG15 is a stable component of mSin3-HDAC complexes, and the MRG15-associated mSin3-HDAC complex also contains Pfl, a component that shares similarity with *S. cerevisiae* Rco1 [25], and a histone H3K4-specific demethylase, RBP2 (KDM5A) [26] (Figure 1(a)). Of note, MRG15 is shared with at least two other complexes: the Tip60 (NuA4)-HAT complex [26–28] and the BRCA2-containing DNA-damage-responsive complexes [29, 30]. Although we will not discuss these complexes further in this paper, the MRG15-associated complexes are implicated in DNA-damage responses [29, 31–33], in addition to transcriptional regulation, suggesting that histone acetylation dynamics is tightly coupled with histone eviction/deposition during DNA-repair processes.

We previously demonstrated that MRG15 recruits RBP2 and controls the H3K4me levels on transcribed regions via the RBP2 activity [26]. In addition, van Oevelen et al. reported that the majority of Sin3 target genes (58%) are bound by RBP2, which spreads over the region immediately downstream of the transcription start site on a subset of E2F target genes during differentiation [34]. These results suggest that the Sin3-HDAC complex and RBP2 play a cooperative role in repressing target genes through deacetylation, demethylation, and, probably, the repositioning of nucleosomes. In *S. cerevisiae*, no evidence has been reported for a physical interaction or functional link between the Sin3 complex and the KDM5 homologue Jhd2. In *Drosophila*, RPD3, MRG15, and Pfl, but not SIN3, were identified in an LID-(a KDM5 homologue) containing complex [35]. Intriguingly, the deacetylation activity of RPD3 is inhibited by its interaction with any of these associated proteins, including LID, implying that the relationship between the HDAC and HDM activities is counteractive.

Recent reports have shown that LID associates with SIN3 and functions in Notch silencing [36]. The Notch signaling pathway plays pleiotropic roles during embryonic development and is important for the regulation of cellular self-renewal [37, 38]. Moshkin et al., purified factors associated with histone chaperones (ASF1 and NAP1) from *Drosophila* S2 cells or embryonic nuclear extract, and identified several peptides, including LID, SIN3A, Pfl, RPD3, MRG15, and a BRCA2-binding protein, EMSY. Using a reciprocal

purification approach, they further showed that NAP1 binds the RPD3 and LID-Associated Factors (RLAFs) consisting of RPD3, LID, SIN3A, Pfl, EMSY, and MRG15, whereas ASF1 binds LAF, a similar complex lacking RPD3 (Figure 1(c)). Both of these complexes, RLAF-NAP1 (RLAF-N) and LAF-ASF1 (LAF-A), are required for the transcriptional repression of Notch-regulated genes. In agreement with the enzymatic activity of LID, the knockdown of ASF1, NAP1, or any of the LAF components results in an accumulation of H3K4me3 at the promoter and enhancer regions. Interestingly, H3K4me3 accumulation is not observed in RPD3 knockdown cells [36]. This implies that LID recruitment to the promoter/enhancer regions of Notch target genes may not depend on the entire RPD3-containing RLAF complex. Considering that the RPD3-containing RLAF complex resembles Rpd3S in *S. cerevisiae* in its subunit composition (Figures 1(b) and 1(c)), it is possible that RLAF acts at the rest of transcribed regions, with or without the association with NAP1. This possibility will be tested by future studies.

RBP2/KDM5A was also found to be involved in the Notch pathway in a study using mammalian cells [39]. RBP-J is a nuclear effector of Notch signaling. Upon ligand binding, RBP-J activates the expression of Notch target genes, and, in the absence of Notch signal, RBP-J switches off the target gene expression. Liefke et al. showed that RBP2/KDM5A associates with RBP-J *in vivo*. RBP2/KDM5A is colocalized with RBP-J at the promoter region of Notch target genes and regulates their expression. Although the involvement of HDAC at the target promoter region was not described in this study, it is most likely that RBP2 works together with an HDAC complex, as in the case of the LAF-A complex in *Drosophila*.

Another function of the Sin3 complex was revealed by a genome-wide study. The gene-expression profiles of Sin3-depleted *Drosophila* cells were analyzed by high-density oligonucleotide array [40]. This analysis revealed that the expression of ~3% of the *Drosophila* genes is altered in Sin3-depleted cells. The affected genes are associated with diverse biological processes, including signal transduction, transcriptional regulation, and cell-cycle control. Interestingly, the list of affected genes also includes a substantial fraction of genes involved in cytosolic and mitochondrial energy-generating pathways. Furthermore, Sin3-deficient *Drosophila* cells exhibit an increase in mitochondrial mass. In accordance with this observation, the genome-wide analysis of RBP2-binding sites using human breast cancer cell lines revealed that RBP2 is enriched at genes encoding proteins that localize to the mitochondrion, including mitochondrial ribosomal proteins [41]. Moreover, the alteration of RBP2 leads to mitochondrial defects in human cells, as in Sin3-depleted *Drosophila* cells.

Overall, the Sin3-HDAC complexes associated with KDM5 regulate the transcription of many genes, in processes such as Notch signaling and mitochondrial functions. Notably, the transcriptional regulation is achieved by dynamic changes in histone modifications not only at promoter regions, but also at transcribed regions. The Sin3-KDM5 complex appears to play an important role at both regions by exchanging its associated cofactors.

3. The Mi-2/NuRD Complex

The NuRD complex was first purified based on its histone deacetylase and nucleosome-remodeling activities [14, 42, 43]. Although the NuRD complex has been identified in mammalian and *Xenopus* cells, its major components have also been found in *Drosophila*, *C. elegans*, and *Arabidopsis*, suggesting that the complex exists widely in animal and plant species. The biochemical association of the histone deacetylase and nucleosome-remodeling activities suggests that these two activities are functionally coupled in this complex action.

The NuRD core complex consists of HDAC1/2, RbAp46, RbAp48, CHD3/CHD4 (Mi-2), MBD2/MBD3, MTA1/MTA2/MTA3, and p66 α /p66 β (Figure 2(a)). HDAC1/2 and RbAp46/48 are shared with the Sin3 complex. Mi-2 was first identified as an autoantigen in the human connective tissue disease dermatomyositis [44], and is a member of the SWI2/SNF2-related chromatin-remodeling ATPases, which target the chromatin region and unwind the nucleosome structure. MBD2 and MBD3 belong to the MBD (methyl-CpG binding) domain family although MBD3 is unable to bind methyl-CpG [45, 46]. Since the NuRD complex can interact with both MBD2 and MBD3, it was first hypothesized to target methylated DNA through its interaction with MBD2 [47]. However, recent reports have shown that MBD3 and MBD2 are exclusively associated with the NuRD complex and form distinct complexes, MBD3/NuRD and MBD2/NuRD [48]. MBD3 and MBD2 are very similar (70% identical), but only MBD3 is essential for mouse development [49].

The MTA protein family has three members, MTA1, MTA2, and MTA3, and vertebrates have two additional splicing variants, small form of MTA1 (MTA1s) and MTA3L [50, 51]. MTA1s, however, lacks a nuclear localization signal and is found in the cytoplasm [50]; it is therefore unlikely to be a component of nuclear Mi-2/NuRD. Among these MTAs, MTA1 and MTA3 have been clearly shown to be involved in tumor progression. Their expression is upregulated in several types of tumors, and their expression states are closely correlated with the invasive growth properties of tumors [51–53]. MTA1 is induced by the growth factor heregulin and is a potent corepressor of estrogen-receptor element-(ERE-)-driven transcription [54]. Similarly, in breast epithelial cells, MTA3 is induced by estrogen, constitutes an estrogen-dependent component of the Mi-2/NuRD complex, and plays a critical role in repressing the expression of Snail, a master regulator of the epithelial-to-mesenchymal transition [51]. These data suggest that MTA proteins are mutually exclusive in the NuRD complex (Figure 2(a)) and that distinct NuRD complexes function as transcriptional repressors in different signaling pathways.

The NuRD complex plays important roles in development. Mice lacking a functional *Mbd3* gene die prior to midgestation [49]. Embryonic stem (ES) cells lacking *Mbd3* are viable, but they fail to completely silence genes that are expressed before implantation of the embryo [55] and are unable to commit to developmental lineages [56]. Another NuRD component, p66, was first cloned in *Drosophila* as

a genetic modifier of the Wingless signaling pathway. The human paralogs p66 α (GATAD2A) and p66 β (GATAD2B) function synergistically in transcriptional repression, and both bind to MBD2 [57]. Although p66 α is not required for normal blastocyst development or implantation, loss-of-function mutant embryos of p66 α die at around day 10 of embryogenesis [58].

ES cells require several key molecules, such as Nanog and Oct4, for their self-renewal and pluripotency [59]. Nanog-containing complexes were recently purified from mouse ES cells, and several NuRD-related components were identified as Nanog-interacting proteins [5]. Interestingly, the Nanog-interacting NuRD-like complex contains most of the NuRD components (Mta1, Mta2, Hdac1/2, p66 α , and p66 β) but lacks Chd3/4, Mbd3, and RbAp46/Rbbp7 (Figure 2(b)). This NuRD-like unique complex was thus named NODE (for Nanog- and Oct4-associated deacetylase). Without Mbd3, deacetylase activity in the canonical NuRD complex is greatly reduced [47], but the NODE complex contains HDAC activity comparable to that of NuRD complexes. Importantly, Mbd3-knockdown ES cells show different gene-expression profiles than Mta1- or Mta2-knockdown ES cells. Moreover, while Mbd3 loss-of-function mutant ES cells can self-renew, the knockdown of NODE subunits leads to the increased expression of developmentally regulated genes and ES-cell differentiation. These observations support the idea that NODE is functionally distinct from canonical NuRD.

Although the functional interaction between NuRD/NODE and histone demethylases in ES cells is still unknown, cooperative roles of NuRD and LSD1 have been described (Figure 2(a)) [60]. Using HeLa cells, Wang et al. purified the MTA2-containing protein complexes and identified LSD1 in addition to other NuRD components [60]. LSD1 interacts directly with all three MTA proteins and appears to form distinct complexes. The NuRD-LSD1 complexes target distinct, yet overlapping, sets of genes. Transcriptional analyses have revealed that these complexes regulate several cellular signaling pathways, including TGF β , cell-communication, focal-adhesion, MAPK, and cell-cycle pathways, which are critically involved in cell growth, survival, migration, and invasion. Furthermore, LSD1 inhibits the invasion of breast cancer cells in a Mi-2-dependent manner *in vitro* and suppresses breast cancer metastatic potential *in vivo*. In fact, LSD1 is downregulated in breast carcinomas and negatively correlated with TGF β 1 expression.

Collectively, the LSD1/NuRD complex is required for the repression of important genes in cellular signaling pathways and suppresses breast cancer metastasis. While LSD1 is a component of NuRD complexes, it has been also observed that LSD1 targets promoter regions independent of NuRD [60]. Therefore, it is likely that LSD1 also functions as a component of currently unidentified protein complexes.

4. The CoREST Complex

CoREST was first identified as a corepressor of REST (RE-1 Silencing Transcription Factor, also known as NRSF) [61] and later demonstrated to be a component of

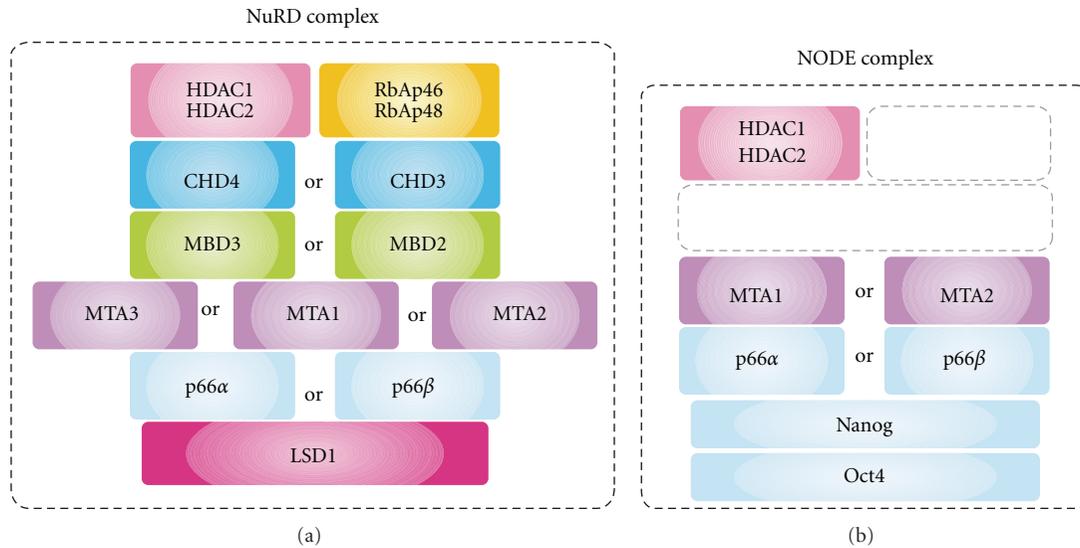


FIGURE 2: *The NuRD complexes.* Schematic representation of the NuRD complex (a) and ES cell-specific NODE complex (b). The empty dotted boxes indicate missing subunits compared with the NuRD complex.

HDAC1/2-containing complexes [62]. The CoREST complex contains HDAC1/2, p80, Sox-like protein, ZNF217 (p110a), and LSD1 (p110b) (Figure 3(a)). Although p110b was identified in the first purification study, its catalytic activity was later identified and renamed LSD1 [6]. Because the CoREST complex does not associate with mSin3, MTA, CHD4, or RbAp46/48, it is thought to be distinct from the other HDAC-containing complexes. The CoREST protein contains two SANT domains, a conserved domain resembling the DNA-binding domains of Myb-related DNA-binding proteins. This domain was originally identified in SWI3, ADA3, NCoR, and TFIIB (and thus named SANT) [63], and it is also present in the MTA proteins. Interestingly, the demethylase activity of LSD1 to the nucleosomal substrates requires the CoREST protein [64]. In addition, the CoREST/LSD1 complex shows high demethylase activity for a hypoacetylated nucleosome substrate, and the SANT domain of CoREST preferentially interacts with hypoacetylated histone tails, as was also previously observed for the SANT domain of SMRT [65]. These results suggest that the HDACs in the CoREST/LSD1 complex function upstream of LSD1 and that deacetylated states are recognized by CoREST, which facilitates LSD1's enzymatic activity.

ZNF217, the other CoREST complex component, is a Krüppel-like zinc-finger protein. The ZNF217 complex purified from HeLa cells includes LSD1, CoREST, HDAC2, and CtBP [66]. The zinc-finger motif of ZNF217 specifically binds to the DNA sequence CAGAAY (Y means C or T), and this consensus is highly conserved in the *E-cadherin* promoter. In fact, ZNF217 and other CoREST complex components are found on the *E-cadherin* promoter, which is repressed by ZNF217. As ZNF217 overexpression has been noted in many cancer cell lines [66], aberrant protein levels of ZNF217 may cause unregulated targeting by the CoREST-LSD1 complex, with a profound effect on cancer progression.

The transcriptional corepressor CtBP (C-terminal binding protein) is also implicated in tumorigenesis. Immunoaffinity purification of the CtBP complex revealed that it contains all of the CoREST complex components [67]. In this analysis, many other interactors were also identified (G9a, HuHMT, HPC2, REBB-1, and ZNF516) [67]. As described for ZNF217, CtBP and the complex components, EuHMT and G9a, also repress *E-cadherin* promoter activity. Another report showed that Snail, a master regulator of the epithelial-to-mesenchymal transition, forms a complex with LSD1 and CoREST, and functions to recruit LSD1 activity to the *E-cadherin* promoter [68]. Together, these results suggest that the CoREST complex is a kind of "core complex" that associates with the CtBP complex as an effector module (Figure 3(a)). Considering that CoREST also associates with other corepressors required for hematopoietic differentiation, Gfi-1 and Gfi-1-b [69], it is likely that CoREST forms several distinct complexes depending on the cellular situation.

5. The NCoR/SMRT Complex

Nuclear hormone receptors are evolutionally conserved, ligand-dependent transcription factors that influence the biological processes of cell proliferation and differentiation in metazoans. NCoR (Nuclear receptor CoRepressor, also known as NCOR1) and SMRT (Silencing Mediator for Retinoid and Thyroid receptor, also known as NCOR2) bind to nuclear hormone receptors and act as "platform proteins" in recruiting a large protein complex that includes Class I and II HDAC and mSin3A [70]. Of note, proteins homologous to NCoR or SMRT are not found in the yeast genome, as is the case for the nuclear hormone receptors. These proteins appear to have arisen during the evolution of metazoan organisms to allow unliganded nuclear hormone receptors

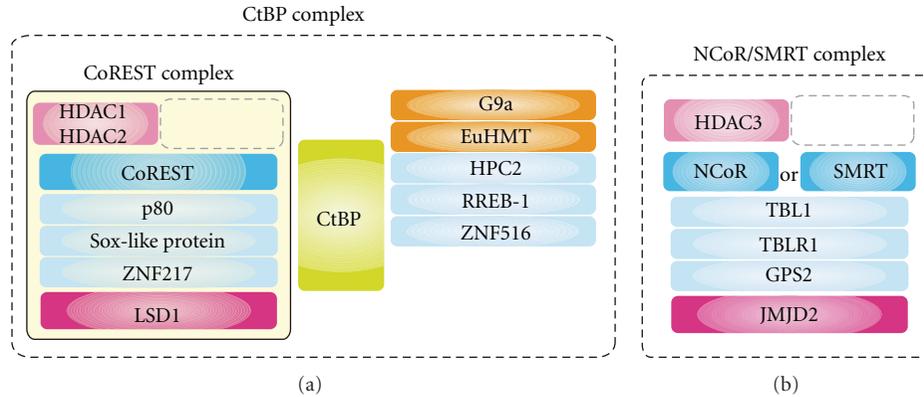


FIGURE 3: *The CoREST and NCoR/SMRT complexes.* Schematic representation of the CoREST (a) and the NCoR/SMRT (b) complexes. The CoREST complex is included in the CtBP-containing complexes. The empty dotted boxes indicate that both CoREST and NCoR/SMRT complexes lack RbAp46/RbAp48 subunits, which are widely observed in HDAC-containing complexes.

to repress transcription via the Sin3-HDAC pathway [70]. SMRT and NCoR share a similar domain organization and are thought to be paralogs.

NCoR and SMRT are essential for development, but their mutant phenotypes are quite different. NCoR appears to be critical to neural differentiation and the developmental progression of erythrocytes and thymocytes [71, 72], whereas SMRT is required for heart development [72]. The SMRT and NCoR complexes share interacting partners, including HDAC3, TBL1 (transducin β -like 1), TBLR1 (TBL related 1), and GPS2 (G-protein pathway suppressor 2) (Figure 3(b)) (reviewed in [73]). Moreover, 114 and 98 proteins, respectively, are reported to interact physically with NCoR and SMRT (BioGRID). NCoR and SMRT also associate with other HDAC members, HDAC4, HDAC5, and HDAC7, but these enzymatic activities are dependent on the SMRT/NCoR-HDAC3 complex [74]. Previous studies demonstrated that diverse transcription factors, including Mad/Mxi, BCL6/LAZ3, ETO, RBP-J, and REST/NRSF, interact with the NCoR and SMRT complexes [75, 76].

Several demethylases that interact with NCoR have been identified. KDM4A (former name, JMJD2/JDM3A/JMJD2A/KIAA0677) was identified in an affinity-purified NCoR complex [77]. KDM4A is not a core subunit of the NCoR complex, and it is not required for NCoR-mediated repression by thyroid hormone receptor. However, KDM4A is required for selective repression of the *ASCL2* gene, which is an imprinted gene essential for proper placental development, and this repression requires a functional NCoR complex [78]. KDM4A specifically demethylates tri- and dimethylated lysine-9 and lysine-36 of histone H3 (H3K9/36 me_{3/2}) [79]. A recent study showed that KDM4 also demethylates lysine-26 of histone H1.4 (H1.4K26me), which is one of seven somatic H1 isoforms in humans, and is known to cause transcriptional repression [80]. Considering that both H3K9me_{3/2} and H1.4K26me are repressive histone marks, it is unclear how KDM4's enzymatic activity is coupled with NCoR-mediated repression. Nevertheless, the substrate specificity of KDM4 may be modulated within N-CoR-containing complexes.

Further studies are required to elucidate the function of the KDM4-associated NCoR complex and the resulting histone-modification dynamics.

KDM5C (also known as SMCX/JARID1C) has histone H3K4-specific demethylase activity and functions as a transcriptional repressor. KDM5C is implicated in X-linked mental retardation, epilepsy, and autism [81–83]. Tahiliani et al., purified a KDM5C-containing complex from HeLa cells and identified NCoR [84]. This complex also contains HDAC1/2, the H3K9-specific methyltransferase G9a, REST (RE-1 Silencing Transcription factor, also known as NRSF), which represses neuronal genes in non-neuronal tissues, along with other proteins that are primarily involved in transcriptional repression. REST not only maintains the transcriptional silencing of a range of neuronal genes in differentiated non-neuronal cells but also plays key roles during lineage commitment in neurogenesis (reviewed in [85]). Tahiliani et al., further showed that KDM5C functions at a subset of REST-regulated neuronal specific genes [84]. It should be noted that NCoR was previously shown to form a stable complex with HDAC3, TBL1, TBLR1, and GPS2 [73], but these proteins were not identified in the KDM5C-containing complex. Thus, it is not clear how NCoR functions in the KDM5C-REST complex. However, a requirement for NCoR in astroglia differentiation was clearly demonstrated [71]. REST modulates not only neuronal, but also glial lineage elaboration [86]. Therefore, it is possible that a unique NCoR-SMCX-REST complex specifically functions in glial development.

Despite its similarity to the NCoR complex, there is no report showing a direct interaction between the SMRT complex and HDMs. However, SMRT appears to control HDM function in trans. SMRT knockout mice exhibit embryonic lethality, with impaired neural development as well as heart defects [72]. SMRT represses the expression of the gene encoding an H3K27-specific demethylase KDM6B (JMJD3), which is also a direct target of the retinoic acid receptor. Retinoic acid induction leads to the release of SMRT from the *KDM6B* gene promoter, and then KDM6B derepresses neurogenic genes in neural stem cells. This finding uncovered

a novel hierarchical relationship between the activities of SMRT and HDM in maintaining the neural stem-cell state.

6. Conclusion

Many HDACs do not function by themselves but act as a component in a multiprotein complex. This complex formation appears to help each HDAC to exert its catalytic activity more effectively and/or specifically. As described above, most HDAC-containing complexes are associated with histone demethylases (HDMs), and the cooperative action of these enzymatic activities enables the complex to act on multiply modified histones at transcriptionally repressive gene regions. Incorporating subtype components gives rise to variations that allow these complexes to repress a wide variety of target genes. Below, we summarize the points raised in this paper.

- (1) The two mammalian Sin3 complexes have distinct functions. In embryonic development, mSin3A is required during the preimplantation stage, whereas mSin3B is required in late gestation. In particular, mSin3B is essential for erythrocyte and granulocyte differentiation.
- (2) *S. cerevisiae* has two Sin3-containing complexes, Rpd3L and Rpd3S. Rpd3L is required for transcriptional repression at gene promoter regions, whereas Rpd3S is located at transcribed regions and prevents spurious transcription from incorrect transcription start sites.
- (3) The *Drosophila* KDM5 protein LID is associated with the SIN3 complex. The LID-SIN3 complex functions to repress the Notch-signaling pathway. Similarly, the human KDM5A protein, RBP2, is required to repress genes of the Notch-signaling pathway.
- (4) Two types of MBD protein, MBD2 and MBD3, are distinctively involved in the NuRD complex. Mbd3/NuRD, but not Mbd2/NuRD, is essential for mouse development. Another NuRD component, MTA1/MTA2/MTA3, also contributes to produce distinct complexes. NuRD complexes with each MTA protein bind to specific promoter regions.
- (5) Mouse embryonic stem cells contain a specialized NuRD-related complex, NODE. The stem-cell-specific factors Nanog and Oct4 are associated with the NODE complex, which represses several differentiation marker genes.
- (6) LSD1 (KDM1A) is associated with the NuRD complex. There are three distinct LSD1-NuRD complexes, LSD1/MTA1/NuRD, LSD1/MTA2/NuRD, and LSD1/MTA3/NuRD. These three different complexes target distinct yet overlapping sets of genes.
- (7) LSD1 is also associated with the CoREST complex. Furthermore, all the CoREST complex components are included in the CtBP-containing complex. Many of these components are required for the repression of the *E-cadherin* promoter.

- (8) NCoR/SMRT is the transcriptional repression machinery for nuclear-receptor-mediated transcription. NCoR has critical roles in neural differentiation and hematopoiesis, whereas SMRT is required for heart development. KDM4A is associated with the NCoR complex, and its association is required for *ASCL2* gene repression. The NCoR complex also binds SMCX (KDM5C), and the NCoR-SMCX-REST complex functions in glial development.

The transcriptional repression machinery is highly elaborate, and we still lack a complete picture of the transcriptional repression mechanisms. Future studies will continue to uncover these complicated mechanisms.

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Research Article

Does Valproic Acid Induce Neuroendocrine Differentiation in Prostate Cancer?

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Valproic Acid (VPA) is a histone deacetylase inhibitor that holds promise for cancer therapy. Here, we investigate whether VPA treatment induces neuroendocrine differentiation of Prostate Cancer (PCa). A tissue microarray of VPA-treated and untreated tumor xenografts and cell lines of human PCa (LNCaP, C4-2, DU145, and PC-3) were generated and were analyzed by immunohistochemical analysis (IHC) for NE markers chromogranin A (CgA), synaptophysin, and NCAM (neural cell adhesion molecule). Western blot analysis for CgA was performed to confirm the results of the TMA. IHC analysis did not reveal any induction of CgA, synaptophysin, or NCAM in any xenograft after VPA treatment *in vivo*. *In vitro*, VPA treatment induced little synaptophysin expression in C4-2 and PC-3 cells and NCAM expression in LNCaP and PC-3 cells. In the case of CgA, VPA treatment decreased its expression *in vitro* in a dose-dependent manner, as determined by western blot analysis. Thus our data demonstrates that VPA does not induce NE differentiation of PCa cells in the physiologically relevant *in vivo* setting.

1. Introduction

Histone acetylation and deacetylation by histone acetyltransferases and histone deacetylases is involved in the epigenetic regulation in human cells [1, 2]. Recently, this post-translational modification has become a popular molecular target for cancer therapy. HDAC inhibitors (HDACIs) have demonstrated significant antitumor activity by hyperacetylation of nucleosomal histones resulting in reexpression of repressed genes that produce growth arrest, terminal differentiation, and/or apoptosis in carcinoma cells [3]. Valproic Acid (VPA), an HDACI and an antiepileptic agent, causes marked decrease in proliferation of Prostate Cancer (PCa) cells *in vitro* and significant reduction in tumor volume *in vivo* [4, 5]. Multiple pathways including cell cycle

arrest, apoptosis, angiogenesis, and senescence contribute to the antitumor effects of VPA.

Neuroendocrine (NE) cells are the third and minor epithelial cell type in prostate, in addition to the more abundant luminal secretory cells and basal cells [6]. NE cells have dual properties of neurons and endocrine cells and are believed to be involved in the regulation, secretion and differentiation of other prostatic cells [7]. Conventional adenocarcinoma with focal NE cells represents the most common type of PCa. Small cell PCa and prostatic carcinoid are relatively rare and are considered pure NE tumors with a poor prognosis [8]. Neuroendocrine differentiation thus has been suggested as a poor prognostic sign by some authors, but the exact role of NE differentiation of the prostate remains unclear, and its prognostic importance

in prostate cancer still remains controversial [7, 9]. The characteristics of NE differentiation in PCa are very much similar to those seen in patients who develop this histologic phenotype in non-small-cell lung cancer [10]. NE cells in prostate express NE markers such as Chromogranin A (CgA), synaptophysin, B-tubulin, neural cell adhesion molecule (NCAM or CD56), neuron specific enolase (NSE), and so forth. NE cells can be generally identified by electron microscopy or immunohistochemical (IHC) staining with antibodies for NE markers [11].

Recently, some studies have documented increased neuroendocrine markers after *in vitro* treatment of prostate cancer cell lines with HDACIs [9, 12] indicating neuroendocrine transdifferentiation. In contrast, studies done in neuroendocrine tumors such as carcinoid, pheochromocytoma, and small cell lung cancers have shown VPA and other HDACIs to exert antitumor effects [13–15]. VPA has been shown to promote apoptosis, reduce NE phenotype and expression of NE markers, and is suggested as a promising therapy for these tumors [16, 17]. Thus the role of HDACI's in neuroendocrine differentiation still remains unclear and has thus warranted further investigation.

The goal of this study is to carefully determine whether VPA induces NE differentiation in the PCa cell lines, *in vivo* and *in vitro*, by studying a variety of markers associated with NE differentiation in numerous PCa cell and tumor models. Markers including CgA, synaptophysin, and NCAM were quantified by IHC in a tissue microarray (TMA) format from several VPA-treated human PCa cells grown *in vitro* and *in vivo* as tumor xenografts in nude mice.

2. Materials and Methods

2.1. Tumor Cell Lines. Human prostate cancer cell lines LNCaP, PC-3, and DU145 were obtained from American Type Culture Collection (Manassas, VA), and C4-2 line was a gift from Dr. Leland Chung (Emory University, Atlanta, GA). All the cells were grown in RPMI 1640 with L-glutamine (Cellgro, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Inc., Carlsbad, CA), 5 $\mu\text{g}/\text{mL}$ ciprofloxacin hydrochloride (U.S. Biological, Swampscott, MA), and 50 $\mu\text{g}/\text{mL}$ gentamicin (Quality Biological, Inc., Gaithersburg, MD). Cells were allowed to grow until 80% to 90% confluent and harvested with 0.05% trypsin/0.53 mmol/L EDTA (Cellgro, Herndon, VA) before each subsequent passage.

2.2. Establishment of Tumor Xenografts. Cell lines were grown to 80% to 90% confluent and harvested. Cells were resuspended in 1x phosphate-buffered saline (pH 7.4; BioSource, Rockville, MD), mixed 1x with Matrigel (BD Biosciences, Palo Alto, California), and injected (1×10^6 per injection) subcutaneously into the lateral flanks of male athymic *nu/nu* mice. Once palpable tumors were established, animals were randomized into control and treatment arms.

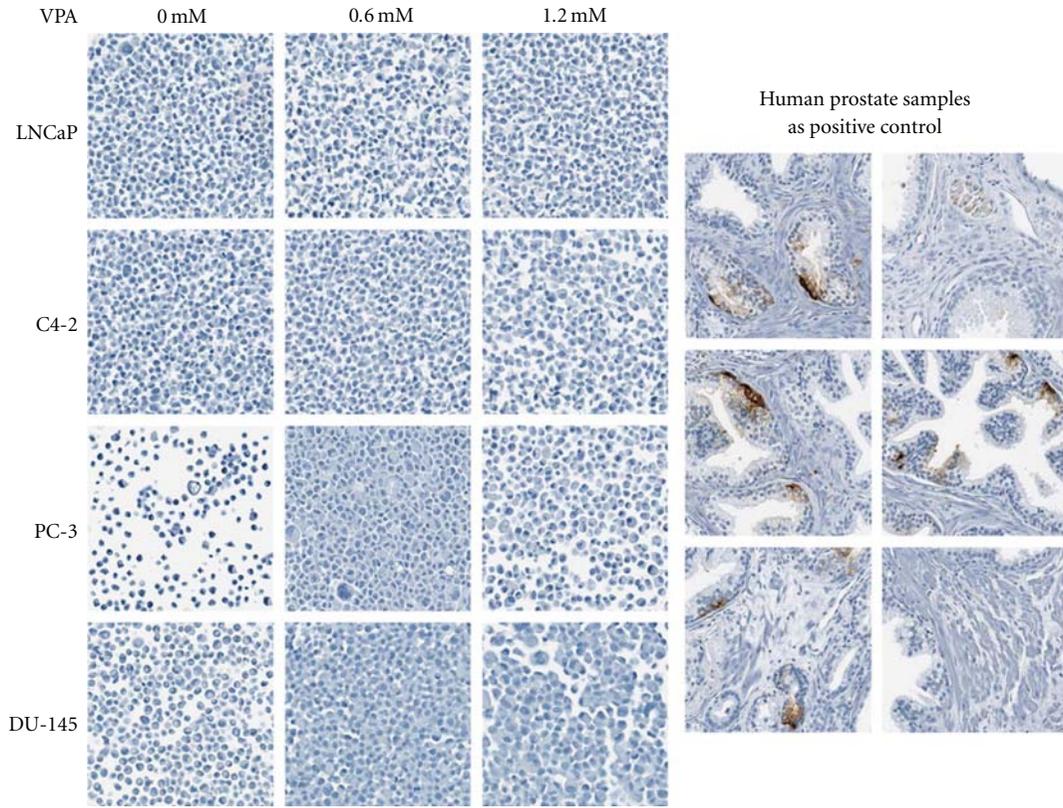
2.3. Valproic Acid Treatment. VPA (1 mol/L; VPA sodium salt; Sigma, St. Louis, MO) stock was made in PBS and filters

sterilized through a 0.22 μm filter. For *in vitro* experiments cell lines were treated with 0, 0.6, and 1.2 mM VPA for 14 days. Medium was replaced every 48 hours with fresh medium containing VPA. For *in vivo* experiments, animals received 0.4% w/v VPA in drinking water. This has been shown to produce blood levels in mouse [4] comparable to FDA approved levels in humans [18]. Animals in treatment arm were treated for 35 days before excision of tumors. In *in vivo* experiments, chronic treatment implies to long-term treatment with regards to life span. We considered 35 days of treatment in nude mice (with life span of 1 year approx. in our experience) as long-term treatment. This period would correspond to years of treatment in humans.

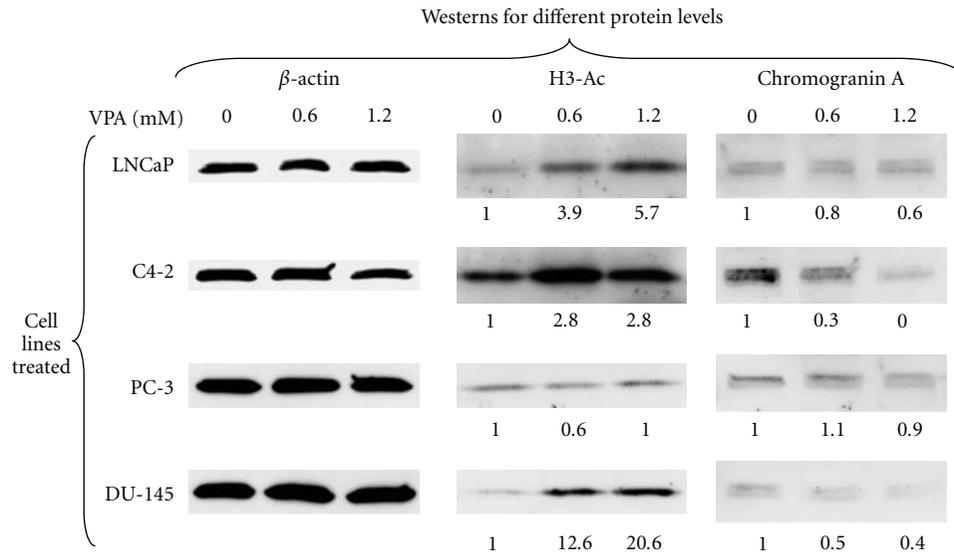
2.4. Western Immunoblotting. Cells treated with different doses of VPA were harvested by trypsinization and resuspended with 5 volumes of cold lysis buffer (RIPA buffer, Cat# R0278, Sigma, St. Louis, MO) and supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Equal amounts of proteins were separated by SDS-PAGE and the resolved proteins transferred to nitrocellulose membrane. The membrane was blocked for an hour in blocking buffer [100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20] with 5% nonfat dry milk and then incubated with rabbit antiacetylated histone H3 (Upstate, Charlottesville, VA) overnight followed by antirabbit IgG peroxidase conjugate (Sigma, St. Louis, MO) for 1.5 hours at room temperature. Immunoreactive bands were detected using the enhanced chemiluminescence plus western blotting detection system (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. Anti-Cip1/WAF1/p21 mouse monoclonal IgG (Upstate, Charlottesville, VA), CgA (LK2H10) mouse monoclonal antibody (Santa Cruz Biotechnology, INC., Santa Cruz, CA), and antimouse IgG peroxidase (Sigma, St. Louis, MO) were used to test p21 and CgA expression separately. Monoclonal anti- β -actin in mouse (Sigma, St. Louis, MO) and antimouse IgG-peroxidase (Sigma, St. Louis, MO) were used to normalize protein loading.

2.5. TMAs: Construction and IHC Staining. For *in vitro* models, cells were harvested and washed in PBS. Resulting cell pellets were incubated for 1-2 hr in Bouin's fixative (75% saturated picric acid, 20% formalin (40%), 5% acetic acid, rinsed with 70% ethanol, and dehydrated according to standard procedures with ethanol and xylene. Cell pellets > 5 mm were split in order to achieve sufficient dehydration. Cells were embedded in paraffin following 90 min of incubation in liquid paraffin at 60°C. For *in vivo* models, tumors were excised and portioned on day 35. Portions were either immediately frozen in liquid nitrogen and stored at -80°C or fixed in buffered formalin and subsequently embedded in paraffin.

A tissue microarray (TMA) of the paraffin embedded materials was generated as described previously [19]. Each array block also contained control normal human prostate tissues and animal xenograft tissues such as bladder, kidney, lung and spleen. Immunohistochemical stains for



(a)



(b)

FIGURE 1: Chromogranin A staining in prostate cancer cells treated with VPA *in vitro*. (a): Representative images of CgA staining for cell pellet sections from control and VPA-treated groups: LNCaP, C4-2, PC-3, and DU-145 (Scanned at 20X magnification using the APERIO imaging system). The tissue specimens of normal human prostate were positive internal controls for CgA staining. (b) Western blot analysis of cells treated *in vitro*. LNCaP, C4-2, DU-145, and PC-3 cells treated with VPA show induction of acetyl-H3, verifying HDACI activity, and downregulation of the NE marker CgA.

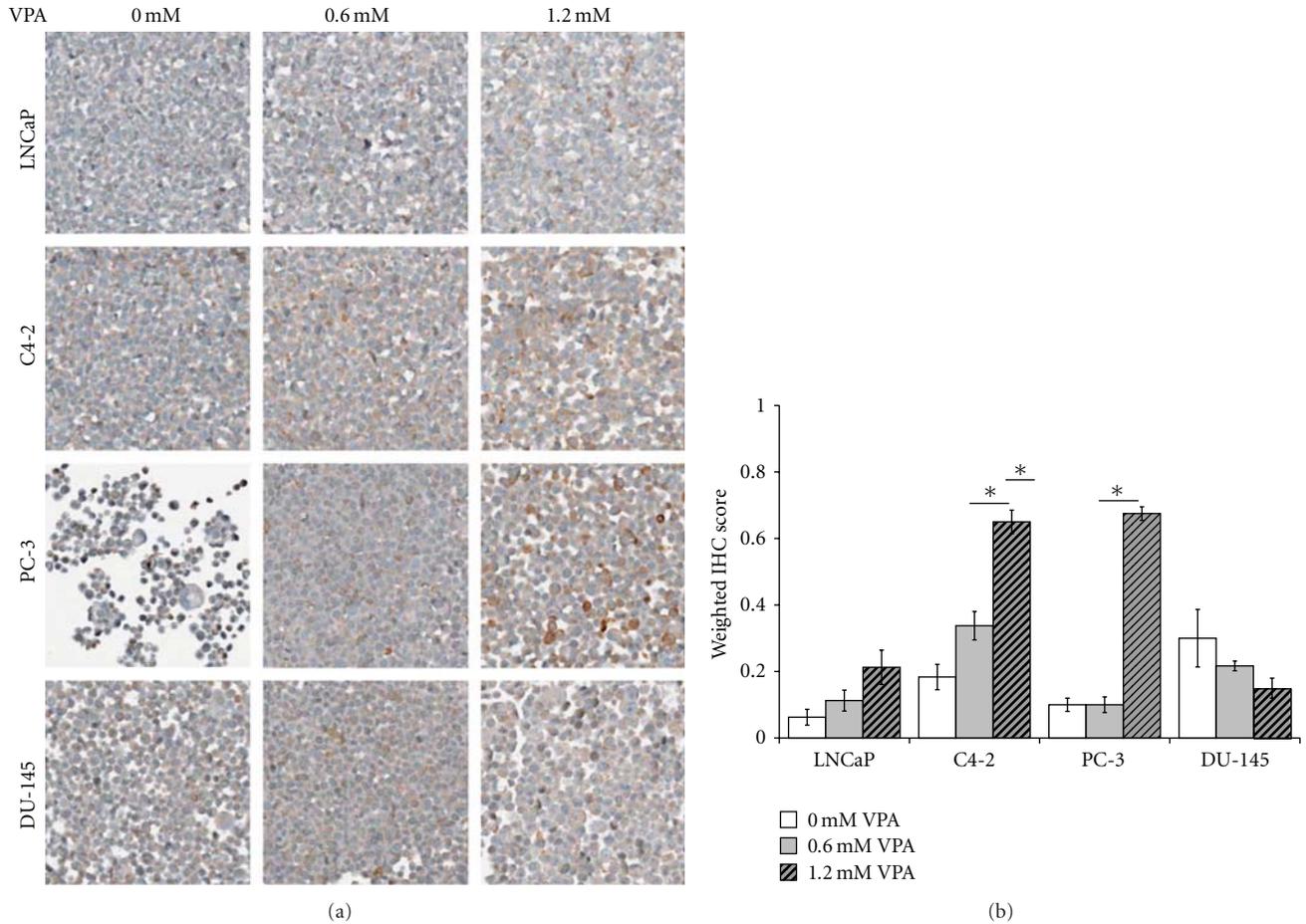


FIGURE 2: Synaptophysin staining in prostate cancer cells treated with VPA *in vitro*. (a): Representative images of synaptophysin staining for cell pellet sections from control and VPA-treated groups: LNCaP, C4-2, PC-3, and DU-145 (Scanned at 20X magnification using the APERIO imaging system). (b) Weighted scoring of IHC staining for synaptophysin (* indicates $P < .05$).

chromogranin A (Clone LK2H10, Ventana, Tucson, AZ), synaptophysin (Polyclonal, Cell Marque, Rocklin, CA), and NCAM/CD56 (123C3.D5, Cell Marque, Rocklin, CA) were performed separately on sections cut from the TMA. Stained TMA slides were scanned (at 20x magnification setting) using the APERIO imaging system and the images were uploaded and viewed using TMAJ [20, 21]. Each array spot was then formed into a composite image for viewing and scoring on a personal computer monitor.

2.6. Scoring of IHC Staining. IHC specimens were provided to pathologist for scoring. The identity of sample and treatment was blinded to the scorer. The specimens showed a varying degree of staining intensity and percentage of cells staining. Therefore, a combined intensity and percentage positive scoring method was used [22]. Strong intensity staining was scored as 3, moderate as 2, weak as 1, and negative as 0. For each intensity score, the percentage of cells with that score was estimated visually. A combined weighted score consisting of the sum of the percentage of cells staining at each intensity level was calculated for each sample, for

example, a case with 50% strong staining, 20% moderate staining, and 10% weak staining would receive a score as follows: $(50 \times 3 + 20 \times 2 + 10 \times 1) = 200$. The maximum score is 300.

2.7. Statistical Analysis. Analysis was done using GraphPad Prism 4.0. Data is plotted as means(SE). One way ANOVA with post-hoc testing was done to evaluate differences in mean staining score between different groups.

3. Results

3.1. In Vitro. The most reliable method to assess NE differentiation in PCa is the detection of CgA in tumor cells. IHC staining for CgA and two other neuroendocrine markers synaptophysin and NCAM were done.

3.1.1. Chronic VPA Treatment Reduces CgA Expression. The IHC staining of sections constructed of cell lines treated for 14 days with VPA was not able to detect any expression of CgA (Figure 1(a)). In order to verify the absence of CgA by

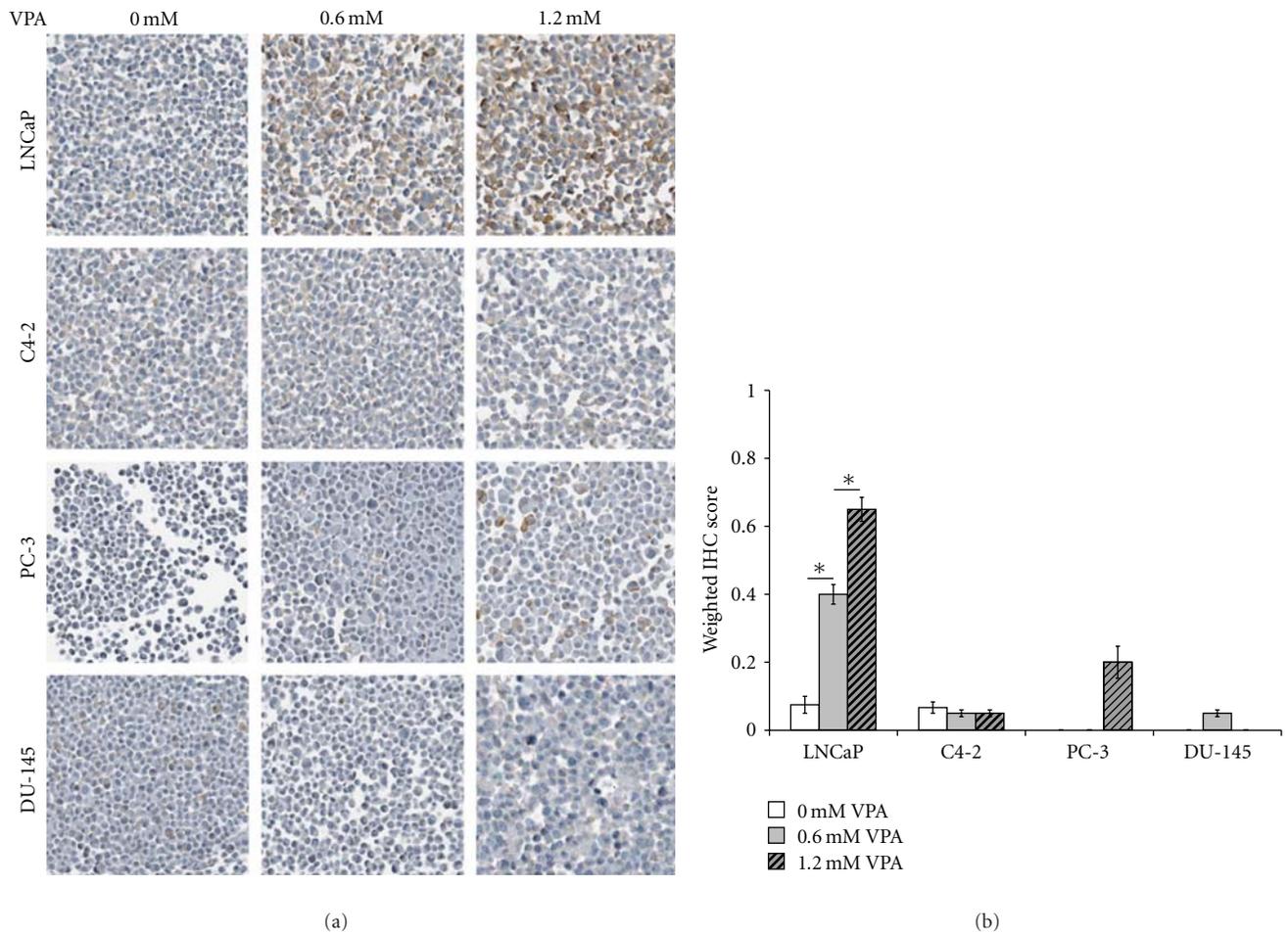


FIGURE 3: NCAM staining in prostate cancer cells treated with VPA *in vitro*. (a): Representative images of NCAM staining for cell pellet sections from control and VPA-treated groups: LNCaP, C4-2, PC-3, and DU-145 (scanned at 20X magnification using the APERIO imaging system). (b) Weighted scoring of IHC staining for NCAM (* indicates $P < .05$).

the IHC in all the cell lines tested, we performed Western blot analysis (Figure 1(b)). Results revealed CgA expression in these cell lines; however, CGA protein levels were reduced in a dose-dependent manner after VPA treatment. Histone 3 acetylation confirmed VPA activity.

3.1.2. Chronic VPA Treatment and Synaptophysin and NCAM Expression. Chronic *in vitro* treatment of C4-2 cells with VPA resulted in increased synaptophysin expression (mean weighted score $65(\pm 4)$ at 1.2 mM versus $34(\pm 4)$ at 0.6 mM ($P = .002$) and $18(\pm 4)$ at 0 mM ($P < .001$)). Significantly increased expression was also found in PC-3 cells treated at 1.2 mM (mean weighted score $68(\pm 2)$ versus $10(\pm 2)$ in other two groups, $P < .001$). However, synaptophysin expression was not altered in Lncap and DU145 cell lines following VPA treatment (Figure 2).

Chronic *in vitro* treatment of VPA increased the expression of NCAM in LNCaP (weighted score $8(\pm 3)$ at 0 mM versus $40(\pm 3)$ at 0.6 mM, ($P < .001$); $40(\pm 3)$ at 0.6 mM versus $65(\pm 4)$ at 1.2 mM, ($P = .002$). In PC-3 cells, no

expression of NCAM was seen at 0 and 0.6 mM VPA, but slight expression was seen at 1.2 mM (mean weighted score $20(\pm 5)$). None or very little NCAM staining was seen in C4-2 and DU145 at either dose (Figure 3).

3.2. In Vivo

3.2.1. Chronic VPA Treatment In Vivo Does Not Induce the Expression of CgA, Synaptophysin, or NCAM. Unilateral tumor xenografts were established in 20 animals each for every cell line. Half of the animals were randomized to receive 0.4% VPA in drinking water. We have shown previously that administration of 0.4% VPA in mouse drinking water can achieve plasma VPA levels similar to the levels obtained in human patients [4]. VPA treatment at these levels was shown to induce acetyl H3K9, p21, and reduce tumor volume, thus confirming the pharmacologic activity of VPA [4]. Animals were sacrificed, and tumors were harvested on day 35. To investigate the effects of VPA on NE markers of PCa tumors *in vivo*, we evaluated expression of NE markers by IHC on the excised tumors. IHC did not reveal any CgA

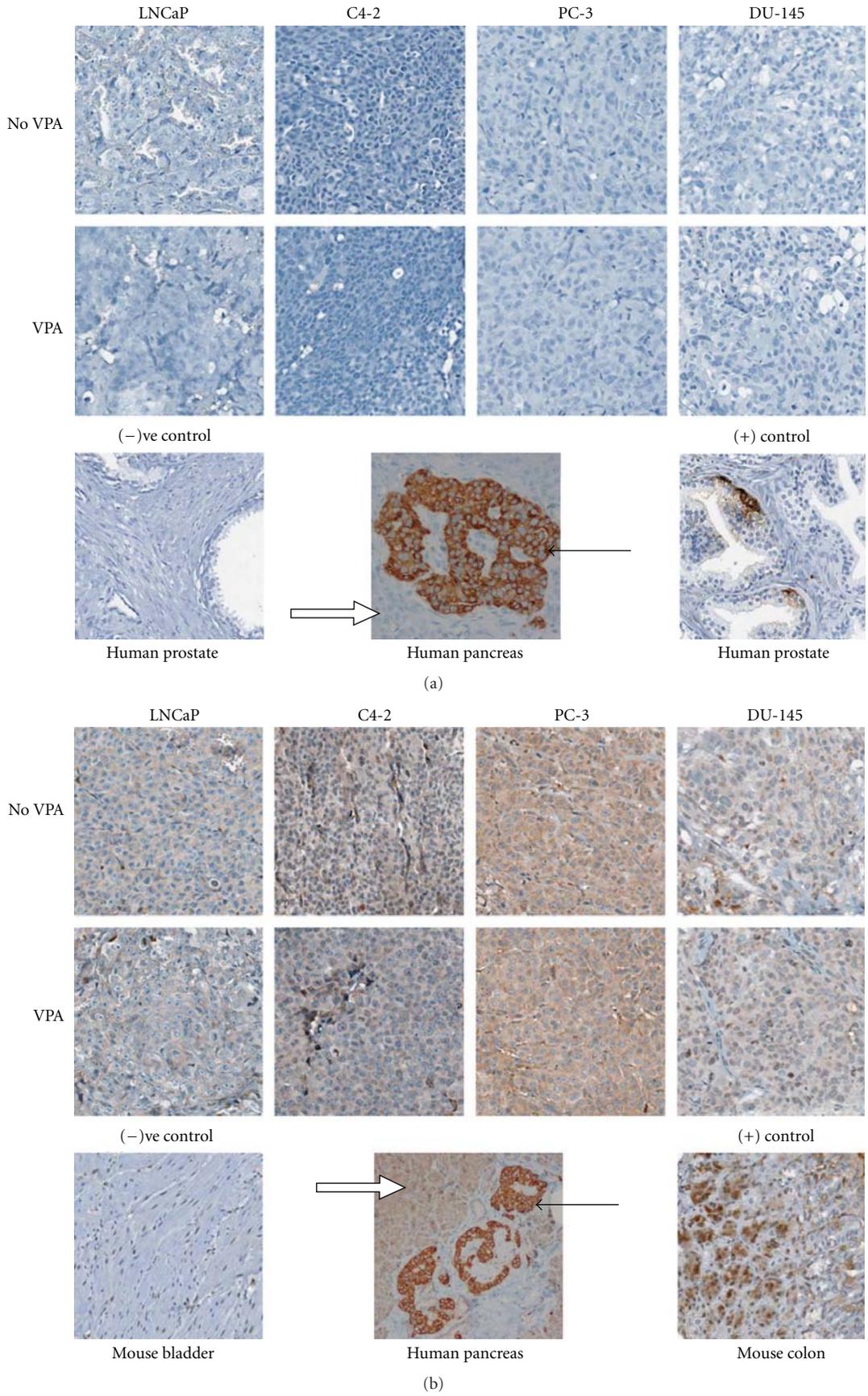


FIGURE 4: Continued.

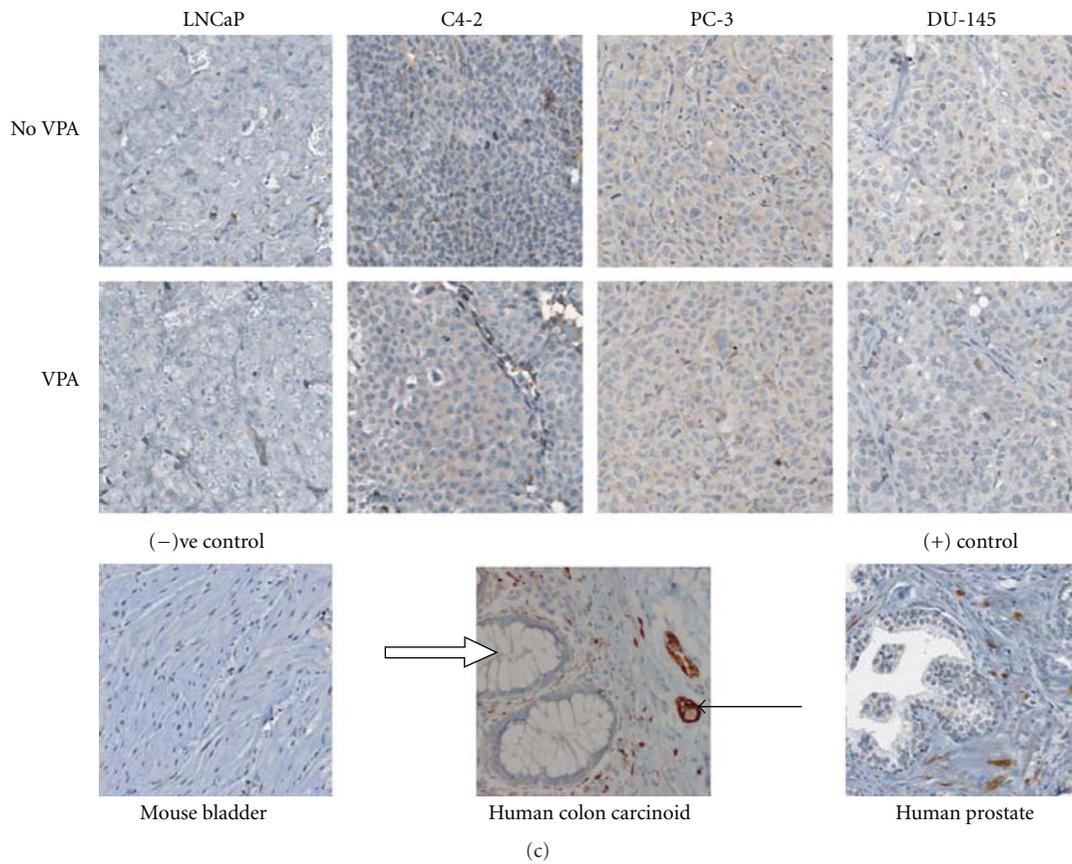


FIGURE 4: Chromogranin A staining in prostate xenografts animals treated with or without VPA. (a): Representative images of CgA staining for xenograft sections from control and VPA-treated groups: LNCaP, C4-2, PC-3, and DU-145 (Scanned at 20X magnification using the APERIO imaging system). The tissue specimens of human pancreas and human prostate were positive internal controls for CGA staining, while another human prostate section served as a negative control. (b) Synaptophysin staining in prostate xenografts animals treated with or without VPA. A: Representative images of synaptophysin staining for xenograft sections from control and VPA-treated groups: LNCaP, C4-2, PC-3, and DU-145 (Scanned at 20X magnification using the APERIO imaging system). The tissue specimens of human pancreas and mouse colon were positive internal controls for synaptophysin staining, while mouse bladder as negative internal controls. (c) NCAM staining in prostate xenografts animals treated with or without VPA. (a): Representative images of CD56 staining for xenograft sections from control and VPA-treated groups: LNCaP, C4-2, PC-3, and DU-145 (scanned at 20X magnification using the APERIO imaging system). The tissue specimens of human colon carcinoid and normal human prostate were positive internal controls for NCAM staining, while mouse bladder was a negative control.

staining in either treatment or control arms in all cell lines (Figure 4(a)). TMAs from C4-2 tumors revealed decreased expression of both synaptophysin (mean weighted score $47(\pm 10)$ versus $15(\pm 5)$, $P < .001$) and NCAM ($44(9)$ versus $5(6)$, $P = .002$) in treatment arms (Figure 4(b)). None of the other arms revealed any significant staining (weighted scores less than 30) for NCAM or synaptophysin (Figures 4(b) and 4(c)). Thus VPA does not induce any NE markers in the physiologically relevant *in vivo* setting.

4. Discussion

NE cells are considered to be derived from local stem cells and are an example of normal, terminally-differentiated cells without proliferative activity [6]. NE cells in tumor lesions are phenotypically similar to NE cells in normal prostate

epithelium in terms of expression of neuropeptides and biogenic amines. Furthermore, dual epithelial characteristics such as prostatic acid phosphatase and/or PSA production and NE marker expression, such as CgA, are frequently coexpressed in the malignant phenotype of NE cells [23]. Studies evaluating the role of focal NE differentiation in PCa prognosis have reported varied results: some reports indicate a negative correlation with prognosis while some show little or no relationship to prognosis [10, 23–28].

Histone deacetylase inhibitors are a promising new class of cancer therapy which have antiproliferative and prodifferentiation properties. For prostate cancer, it was recently reported that HDAC gene expression is elevated in tumors. Moreover, high expression levels of HDAC2 were associated with poor prognosis [29]. Thus VPA, which is capable of inhibiting HDACs classes I and IIa, may be a good

option for PCa therapy. In preclinical models, VPA treatment leads to proliferation arrest and differentiation and apoptosis of cancer cells of various tissue origin, while nominal effects were reported in normal cells [2, 4, 5]. However, Valentini et al. reported VPA to cause an increase in the secretion of NSE in LNCaP cells (*in vitro*) which may indicate an NE differentiation [12]. In order to better understand the role of VPA in possibly stimulating NE differentiation in PCa cell models, we selected the clinically recommended panel of antibodies for the IHC investigation of NE cells in multiple PCa cell lines and xenograft tumors.

Chromogranin A or parathyroid secretory protein 1 is a member of the chromogranin-secretogranin family and forms the major constituent in neurosecretory peptide containing dense core granules in NE cells. CgA is highly expressed by cells of neuroendocrine origin, both normal and tumoral, functional and nonfunctional. While Neuron Specific Enolase (NSE) has also been used as an NE marker, it is known to be expressed in a variety of non-NE cells and tumors, which has led researchers to question its specificity [30, 31]. Serum CgA levels, on the other hand, have been reported to be better predictors of neuroendocrine differentiation than NSE [32, 33]. Thus, CgA now is widely regarded as an excellent and more specific marker of NE differentiation. In our study, CgA expression was not detected by IHC in either control or treated groups in human prostate cell lines of LNCaP, C4-2, PC-3, and DU145 *in vitro* or *in vivo*. Western blotting, being more sensitive, revealed low CgA expression in these cell lines; which reduced further with VPA treatment in a dose-dependent manner. Histone acetylation and p21 induction (data not shown) confirmed that active VPA doses were achieved as we have previously demonstrated [5, 34]. Our results are further corroborated by similar reduction in NE markers and NE morphology in NE tumors after treatment with HDACIs [13, 14, 16]. These studies in fact report apoptotic effects of VPA on neuroendocrine cells. Yu et al. have previously demonstrated CgA to be an important neuropeptide promoting the growth of prostate cancer cells and its suppression leading to programmed cell death in multiple prostate cell lines [35]. Gong et al. later found antiapoptotic effects of CgA to be dependent on a Protein Kinase B/Akt (an antiapoptotic protein or prosurvival factor) mediated pathway [36]. Also HDACIs have been known to downregulate Akt phosphorylation in prostate cancer cells [37]. Taken together, it suggests a link between HDACI's-mediated apoptosis and CgA inhibition. Further studies will be required to determine the contribution of CgA and Akt to the VPA therapeutic effect.

Synaptophysin and NCAM are other specific and fairly sensitive markers for NE differentiation [38, 39]. In *in vitro* experiments, these markers showed varying trends (increased synaptophysin staining in C4-2 and PC-3 cells but unaltered in LNCaP and DU145; increased NCAM in LNCaP and PC-3 cells but unaltered in C4-2 and DU145), and no consistent pattern was seen. In *in vivo* experiments, the staining did not reveal any significant expression of these markers in any of the xenografts except in C4-2 tumors where it revealed a downward trend on treatment.

5. Conclusion

The findings in our study do not support any neuroendocrine differentiating role of VPA. On the contrary, CgA, a very specific marker, was reduced in all studied cell lines, following chronic VPA treatment. Synaptophysin and NCAM showed some inconsistent induction following VPA treatment in some cell lines but, *in vivo*, VPA treatment did not induce any significant expression of any NE markers. 0.4% VPA in mouse drinking water can achieve plasma VPA levels similar to the therapeutic levels obtained in human patients. TMAs from xenografts of different cell lines either did not stain for NE markers or had very little staining without any induction on VPA treatment. Thus, our data clearly demonstrates that VPA does not induce NE differentiation of PCa cells in the physiologically relevant *in vivo* setting.

Acknowledgment

A. Sidana and M. Wang contributed equally to the work.

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Review Article

Rationale for Possible Targeting of Histone Deacetylase Signaling in Cancer Diseases with a Special Reference to Pancreatic Cancer

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There is ongoing interest to identify signaling pathways and genes that play a key role in carcinogenesis and the development of resistance to antitumoral drugs. Given that histone deacetylases (HDACs) interact with various partners through complex molecular mechanisms leading to the control of gene expression, they have captured the attention of a large number of researchers. As a family of transcriptional corepressors, they have emerged as important regulators of cell differentiation, cell cycle progression, and apoptosis. Several HDAC inhibitors (HDACis) have been shown to efficiently protect against the growth of tumor cells *in vitro* as well as *in vivo*. The pancreatic cancer which represents one of the most aggressive cancer still suffers from inefficient therapy. Recent data, although using *in vitro* tumor cell cultures and *in vivo* chimeric mouse model, have shown that some of the HDACi do express antipancreatic tumor activity. This provides hope that some of the HDACi could be potential efficient anti-pancreatic cancer drugs. The purpose of this review is to analyze some of the current data of HDACi as possible targets of drug development and to provide some insight into the current problems with pancreatic cancer and points of interest for further study of HDACi as potential molecules for pancreatic cancer adjuvant therapy.

1. Background

Cancer diffusion and metastasis account for approximately 90% of all cancer-related deaths [1]. Metastasis follows a multistep complex processes in which neighboring healthy tissue is invaded by primary tumor cells, which access the systemic circulation and finally proliferate at distant sites into macroscopic secondary tumors via the perivascular and/or perilymphatic tissue [2]. In the case of pancreatic cancer, most of the patients already have metastases at the time of diagnosis. These patients have a poor prognosis, and less than 5% of patients are alive 5 years after the initial diagnosis [3].

Specific events that promote tumorigenesis and cancer progression are linked with complex molecular modifications such as DNA methylation, histone acetylation,

phosphorylation, ubiquitylation, and ADP ribosylation. Currently, results from basic research underline the importance of acetylation and deacetylation at the level of not only histone lysine residues but also other cellular factors that are supposed to interfere with the regulation of gene expression. HDAC enzymes play a central role to oppose histone deacetylases (HDATs), which catalyze histone acetyltransferases (HATs) [4].

Previously, eighteen mammalian HDACs have been characterized and are currently classified as follows: Class I and II share similarities with yeast deacetylases RPD3 and HDA1; Class III shows homology to yeast silent information regulatory protein (SIR2p) [5]; Class I includes HDACs 1, 2, 3, and 8; Class II comprises HDACs 4, 5, 6, 7, and 9 [5]. HDAC6 and HDAC10 are carriers of two catalytic sites

and are therefore grouped in subclass IIB. HDAC 11 shares conserved residues with Class I and II enzymes in their catalytic site and is allocated to Class IV [5].

Based on their primary structure, the SIR2 family [Hst proteins (Homologous of Sir two)] or sirtuins are currently grouped into five different classes [6]: Sirtuin Class I: Human SIRT1, 2, 3; Class II: SIRT4; Class III: SIRT5; Class IV: SIRT6, 7, SIR-T8, which was recently detected in thyroid carcinoma cell lines and tissue samples [7], shares 85% homology in the core sirtuin domain with SIR-T7 and is therefore grouped into Class IV.

HDAC enzymes differ in their subcellular localization, catalytic activity, and susceptibility to different inhibitors. Class I HDACs are found exclusively in the nucleus, whereas HDAC3 has both nuclear import (NIS) and export (NES) signals being able to localize to the cytoplasm [8]. The absence of NES in HDAC1 and HDAC2 sequences attest to their nuclear localization [8]. HDAC11, the unique member of class IV, resides in the nucleus [5]. While Class II HDACs are able to shuttle in and out of the nucleus, the Class III sirtuin family (SIRT1-7) has a different localization. Whereas three SIRT proteins (SIRT1, SIRT6, and SIRT7) are nuclear localized, SIRT3, SIRT4, and SIRT5 are localized in the mitochondria and SIRT2 is a cytoplasmic protein [8].

HDAC enzymes deacetylate histones and other protein substrates. Trichostatin (TSA), a fermentation product of *Streptomyces*, originally used as an antifungal agent, was found to have anticancer cells proliferation activity and the ability to inhibit HDAC with an IC_{50} in the nanomolar range [8]. In a recent elegant study a high-throughput, precise profiling of HDACi potency against all class I and II enzymes has been achieved using a panel of structurally diverse small HDACi molecules comprising those reported in the literature [9]. Surprisingly, an apparent redundancy of pharmaceutical compounds toward HDAC1, HDAC2, and HDAC3 was evidenced and at relevant concentrations the class IIa enzymes are not targeted by most HDACi tested. Unlike other class I and II HDACs, the sirtuins require nicotinamide dinucleotide (NAD) as a cofactor. The search for sirtuin inhibitors has identified, besides the physiological inhibitor nicotinamide, synthetic inhibitors such as sirtinol and splitomicin [10, 11].

2. HDACs and Cancer

Growing knowledge about HDACs/SIRTs shows that they are regulators of growth, differentiation and cell death (apoptosis). The dysfunction of transcriptional repression mediated by HDACs may lead to carcinogenesis. Indeed, modulation of expression levels of genes encoding HDACs (over- and/or underexpression) has been reported for different types of cancer [5]. For example, overexpression of HDAC1 has been reported in gastric [12] and HDAC2 and HDAC3 in colorectal cancer [13, 14]. Decreased transcription of the *HDAC5* gene has been observed in colorectal cancer [15]. In regards to Class III HDACs, SIRT8 was found to be overexpressed in thyroid cancer [16], while *SIRT2* gene expression is downregulated in human gliomas [17].

Among the targets of HDACs are members of the family of nuclear factors Rel/NF-kappa B. However, NF-kappa B is activated in the early stages of tumor transformation of mammalian cells [18]. Similarly, NF-kappa B is constitutively active in the case of human adenocarcinoma of the pancreas [19] and in leukemic T-lymphocytes [20], but not in breast cancer [21].

It has also been demonstrated that the action of HDACs may modulate the activity of NF-kappa B. Indeed, deacetylation of the RelA/p65 subunit of NF-kappa B by HDAC1, HDAC2 [22], or HDAC3 [23] increases its association with $I\kappa B\alpha$, which leads to a loss of transactivation activity. Furthermore, deacetylation of lysine 310 of Rel/p65 by SIRT1 represses the transactivating activity of NF κ B and consequently its antiapoptotic property [24]. In summary, these data underline a possible involvement of HDACs in the process of tumorigenesis.

3. HDACi and Cancer Therapy/Mechanisms of Action of HDACi

Since the discovery of the anti-tumor effect of Trichostatin TSA in 1990 [25], many other HDACi have been identified. A major therapeutic limitation of HDACi is their nonselectivity. Indeed, they target both HDAC Class I and Class II. Although some HDACi have some degree of selectivity such as the depsipeptide (FK228) with a preference for HDACs 1 and 2 [9], to date, an HDAC isoform selective inhibitor has not been developed which indicates that selectivity may be less of a therapeutic limitation than the so-called pan-HDACi. The mechanism of action was elucidated by crystallization of the catalytic domain of TSA or suberoylanilide hydroxamic acid (SAHA) [26]. TSA is a product based on a hydroxamic acid, which reaches the active site of HDAC, chelates Zn^{2+} , and inhibits the enzyme at nanomolar concentrations [5, 26].

4. Cytostatic Effect and Induction of Differentiation

HDACi induce differentiation of tumor cells. Initial applications have focused on promyelocytic leukemia, in which the expression of the fusion protein PML-RAR plays a crucial role. This part of the RAR (retinoic acid receptor) behaves as a constitutive transcriptional repressor of differentiation; HDACi, which lift the repression, initiate the differentiation program. Similar results were obtained in various models of leukemia and solid tumors. The treatment of such tumors by a variety of HDACi shows that the inhibitors have a cytostatic effect on tumor cells by induction of cell cycle arrest in G1 or in some cases in the G2 phase [27]. The *p21* gene is the target that appears to be most often associated with this phenotype. It is a repressor complex that is formed by cyclins and kinases, cyclin E-Cdk2, and cyclin A-Cdk2 whose constitutive activity is responsible for the deregulation of the cell cycle of tumor cells. HDACi may act, at least in some cases, in synergy with retinoic acid, thereby inducing differentiation of myeloid cells *in vitro* and *in vivo* [5].

5. Induction of Apoptosis

In most cases, HDACi induce death of tumor cells by apoptosis through either the intrinsic or extrinsic pathways [27]. The intrinsic pathway involves the activation of mitochondrial proapoptotic members, which leads to the generation of free radicals. A number of studies have shown that overexpression of Bcl-2 not only inhibits apoptosis, but also inhibits the apoptosis-inducing effect of HDACi. For instance, lymphoma B cells that overexpress Bcl2 were found to be resistant to SAHA [5]. Moreover, in prostate cancer cell line PC3, SAHA causes an increase accumulation of Bcl-2 which correlates with the resistance to SAHA-induced cell death. In contrast, the lack of Bcl-2 in DU145 cell line is associated with its marked sensitivity [28]. In the extrinsic pathway, HDACi can induce expression of Fas and its ligand. The protein death domain TRAIL also seems to be induced in tumor cells derived from acute promyelocytic leukemia [29]. Indeed, in a transgenic mouse model defective in TRAIL receptor expression, treatment with valproic acid (HDACi of Class I) induces overexpression of these receptors. Moreover, their loss reduced the induction of apoptosis by 50% in the transgenic mouse model [30]. Additionally, it has been shown that HDACis have an effect on the cleavage of Bid and phosphorylation of Bad and Bim [31–33]. However, the initiation process is not yet well established. It is possible that Bid is activated via a promoter hyperacetylation and the activation of a transcription factor (E2F1) [34, 35].

6. Effect on the Production of Oxygen Radicals

HDACis induce the production of free oxygen radicals. However, the exact mechanism leading to this phenomenon is unknown [36]. A change of the mitochondrial membrane potential occurs after a rise in free oxygen radicals. However, even within this apoptotic pathway, there are a number of unknown parameters. Indeed, the work of Garcia Morales et al. [37] showed that despite overexpression of caspase 3 after treatment of three pancreatic cancer cell lines with HDACi, the inhibition of caspase 3 did not abolish cell death by apoptosis. These results support the fact that the apoptotic pathways induced by HDACi is still far from being elucidated.

7. Antiangiogenic Activity

The HDACi have antiangiogenic properties due to their ability to repress the expression of VEGF (vascular endothelial growth factor) *in vitro* and *in vivo* [38]. They also suppress the transcription of the chemokine (CXCR4) encoding gene that plays a pivotal role in both the differentiation of endothelial cells in the bone marrow and their mobilization [39]. Moreover, inhibition of HDACs induced by FK228, a cyclic depsipeptide isolated from *Chromobacterium violaceum*, increases gene transcription encoding factors that inhibit angiogenesis and decreases the expression of the gene encoding factors stimulating angiogenesis. These observations demonstrate that HDACis interfere with the process of neovascularization, which is pivotal for tumor growth.

8. Immunomodulatory Properties

HDACis play a role in anti-tumor immune mechanisms. HDACis activate the transcription of genes encoding MHC Class I and II molecules and also costimulate CD40, CD80, and CD86 molecules [40, 41]. This biological property appears to be related to the removal of repression [42]. Furthermore, HDACi can also alter the secretion of different interleukins. For instance, suberoylanilide hydroxamic acid (SAHA) reduces acute graft-versus-host disease after allogeneic bone marrow transplantation [43]. This action results from a decreased production of different interleukins (TNF, INF γ and IL-1), which all play a major role in graft-versus-host reactions. Moreover, it has been shown that the acetylation status of *STAT1* (signal transducer and activator of transcription) [44], *STAT3* [45], as well as NF-kappa B, is related to their degree of activation/deactivation, which affects the cytokine secretion profile [23]. Furthermore, SAHA activates differentiation of dendritic cells [46], which potentiates the immune response against tumors.

A link between inflammation and cancer development has been shown in population studies. Regulatory factors such as NF-kappa B, as described above, are substrates of HDACs [27]. In premalignant cells, NF-kappa B, by inducing the expression of proinflammatory and survival genes and inhibiting those that promote cell death, may increase the potential of precancerous cells. By blocking the transactivation of NF-kappa B, HDACi may promote the expression of genes that are repressed in tumor cells. Therefore, they exert their therapeutic effect by reverting the tumor phenotype of cancer cells to a normal phenotype.

9. Combination of HDACi and Anticancer Drugs

Interestingly, HDACi can potentiate the anti-tumor effect of drugs [27]. It has been previously demonstrated that coincubation of K562 leukemic cells with a proteasome inhibitor, bortezomib, SAHA, or sodium butyrate, increases apoptosis by inducing alteration of mitochondrial function [47]. Bortezomib, which is used for the treatment of myeloma, has a limited action due to formation of aggregates and corresponding accumulation of ubiquitylated proteins. This limiting step is controlled by HDAC6. A recent study demonstrated that the use of TSA, which inhibits the action of HDAC6, enhances bortezomib-induced apoptosis in pancreatic cancer cells [48]. Moreover, pretreatment of human leukemic cells with MS-275, an orally active synthetic benzamide derivative that belongs to HDACi significantly enhances the activity of the cytotoxic drug Fludarabine, a purine analogue that has demonstrated significant activity in B-cell malignancies [49].

10. Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) is ranked fourth and fifth to sixth leading cause of cancer death in USA and Europe, respectively [50]. Standard treatments for

advanced disease include radiotherapy and/or chemotherapy regimens. However, radiotherapy is often toxic and the chemotherapy which includes drugs such as 5-fluorouracil (5-FU) and gemcitabine (GEM) are either ineffective or effective for only short duration [51]. Recent phase III trial comparing the efficiency of treatment with either GEM alone or GEM combined to Erlotinib, an inhibitor of EGFR tyrosine kinase showed a modest increased survival time of 15 days [52]. Furthermore, a combination of GEM with the bevacizumab, an inhibitor of VEGF, in a phase III trial did not increase survival time [53]. Moreover, a failure to demonstrate significant clinical benefit for the patients was recorded in a phase III trial combining GEM and cetuximab, an anti-EGFR monoclonal antibody [54].

Historically, the ras onco-proteins have been considered the target substrate responsible for the antiproliferative effects of enzyme farnesyl protein transferase (FPT) inhibition. Despite 70 to 90% mutation of *K-ras* in pancreatic cancer [55], the combination of FPT inhibitor and gemcitabine did not prolong overall survival in advanced pancreatic cancer compared with single agent gemcitabine [56]. The reasons for the failure of these treatments are not yet understood.

Tumor chemoresistance could be among factors responsible for the lack of effective therapies [57]. Indeed, multiple biochemical and molecular alterations occur in cancer cells. For instance, pancreatic cells overexpress the death receptor decoys DcR2 and DcR3 as well as BcL-XL which play a role in pancreatic cancer chemoresistance [57]. Moreover, a number of other genetic alterations for several specific genes including *p53*, *p16^{INK4a}*, and *Smad4*, have been documented [58]. Interestingly, reintroduction of the wild-type *p53* in the human pancreatic cells increases their sensitivity to GEM [59].

Therefore, in pancreatic cancer, there is an urgent need for rationally designed molecules displaying improved efficacy and tolerability compared to existing treatments. HDACi have emerged as promising antineoplastic agents. In human pancreatic adenocarcinoma cell lines, TSA and SAHA have been shown to induce the cell death by apoptosis found to be caspase independent [60]. Other investigators have reported that TSA can synergize with gemcitabine [61] or the proteasome inhibitor PS-341 [62] to induce apoptosis of human pancreatic cancer cells. In a recently published study by our research group, we demonstrated that HDAC Class I and II inhibitors such as TSA can induce death of tumor cell lines via apoptosis [63]. Interestingly, the data also showed, for the first time, that Class III HDACis, such as sirtinol and nicotinamide, are able to induce pancreatic cell death. However, whether sirtuin inhibitors are efficient anticancer drugs *in vivo* is yet to be demonstrated.

To provide insight into the biological behavior of pancreatic cancer and to identify new potential biomarkers, a study aiming to examine the levels of *HDAC* and *SIRT* gene expression in pancreatic cancer compared to normal pancreas tissue has been initiated. Because normal pancreatic tissue can only be obtained under certain circumstances (i.e., donor liver transplantation), samples of control tissues from the surgical specimens of patients with pancreatic

adenocarcinomas [close proximity termed normal adjacent, far away from the tumor as possible termed normal distant], serous cystadenoma (SC), intraductal papillary mucinous tumor of the pancreas (IMP), or complicating chronic pancreatitis (CP) have to be used as controls. So far, an increased number of *SIRT5* mRNA transcripts have been observed not only in most of the Pancreatic adenocarcinoma (PA) samples but also in other tissues samples (SC, IMP and CP). Approximately 81% of the PA tissue samples displayed increased expression of *HDAC7* mRNA transcripts as well as its corresponding protein [64]. Furthermore, it has been shown that more than 90% of the analyzed PA samples contain activated point mutations of the *K-ras* gene, and a large number of these neoplasms also exhibit alterations in genes controlling the G1/S-phase cell cycle transition such as *p16^{INK4a}* [65]. Additionally, the *p16^{INK4a}* genetic alterations are significantly more frequently observed in patients with the shortest tumor survival compared with those patients with the longest [65]. Other molecular alterations may contribute to carcinogenesis of the pancreas such as those related to growth factors and/or their receptors. Due to the fact that almost 90% of the patients are unsuitable for resection, our own data are the first to demonstrate that HDAC gene expression, particularly HDAC7, could be a possible marker of PA [64]. Although it is difficult to determine at this stage whether upregulation of HDAC7 in PA is a cause or a consequence of malignant progression, its overexpression in cancerous tissues and not in their normal counterparts constitutes an interesting field of future research for new approaches in the design of antipancreatic cancer therapy.

HDACs are key modulators of endothelial cell migration and angiogenesis and regulate PDGF-Bp/PDGF-beta gene expression [66]. Taking into account that angiogenesis is required for tumor progression, it is reasonable to suggest that molecules that are able to interfere with HDAC expression/activity may be of particular therapeutic benefit. In this perspective, a recent study has shown that vorinostat, the first HDACi approved for clinical trials in the treatment of cutaneous T-cell lymphoma [67], selectively downregulates HDAC7 expression [68]. However, rational targeting of HDAC7 signaling will require extensive investigation of cross-talk between the HDAC7 signaling pathway and other pathways involved in pancreatic cancer progression. This will probably allow us to define which component will be more effective when combined with a potential HDAC7 inhibitor. Another field nicely developed in a recent review by Stimson and La Thangue is the search for predictive biomarkers that may inform on the tumor response to HDACi [69]. For instance, the *HR23B* gene validated as a sensitivity determinant for HDACi-induced apoptosis, was identified at high levels in cutaneous T-cell lymphoma (CTCL) *in situ*, a malignancy that responds favorably to HDACi-based therapy. Therefore, the identification of such biomarkers which might be linked to HDAC7 overexpression will help to select patients who might benefit from HDACi therapy. A recent study have shown that histone modification levels indicate patients treated for pancreatic cancer which adjuvant chemotherapy were more or less likely to derive survival

benefit from adjuvant fluorouracil relative to gemcitabine. Although differences were modest and require validation, they raise the possibility that histone modification levels could serve as predictive biomarkers for adjuvant treatment [70]. For instance, it has been previously demonstrated that HDAC7 interacts with the transcriptional regulator MEF2D, which binds to the promoters and transcriptionally represses the proapoptotic orphan receptor Nur77 [71]. Interestingly, treatment of CTCL with panobinostat (LBH589, Novartis Pharmaceuticals Basle, Switzerland) inhibits the mRNA and protein levels of HDAC7 and induces expression and translocation of Nur77 to the mitochondria where it converts death resistance protein BCL-2 into a killer protein, therefore leading to the death of cultured and patient-derived human CTCL cells [72]. Furthermore, the majority of HDAC7 are localized in the nucleus; however, despite the absence of NES signal, it is also found in the cytoplasm. The regulation of nucleocytoplasmic shuttling is not yet clearly defined, although studies have shown that the cellular concentration of factors such as 14-3-3 (a cytosolic anchor protein), CaMKI (Ca²⁺/calmodulin-dependent kinase), and other yet unknown molecules may determine the subcellular localization of HDAC7 in a cell type and HDAC-specific manner [73]. Others have reported that the activity of HDAC7 in the nucleus is dependent upon its interaction with HDAC3. Consequently, cytoplasmic HDAC7, which is not bound to HDAC3, is enzymatically inactive [74]. Taken together these observations pointed to the need of further investigations of pancreatic-tumor-associated HDAC7 in order to better understand its exact role in cancer progression and to design selective inhibitors containing a variety of scaffolds with interesting physicochemical properties.

11. Critical Point of View

The critical challenge in pancreatic cancer is the detection of early lesions at an asymptomatic stage that will allow for curative resection and to offer a greater chance of a cure. In recent years, HDACs emerged as promising targets for therapeutic interventions that could revert the aberrant epigenetic states associated with cancer [75, 76]. Thus, in recent years HDACs have being leading focus for the clinical development of molecules that modulate their activities. A number of HDACi have been identified, they represent promising additive in cancer therapy as they can induce upregulation of specific proapoptotic genes and/or downregulation of prosurvival genes, therefore reactivating pathways controlling apoptosis, differentiation, or cell growth [27]. In recent *in vitro* studies, HDACi inhibitors such as TSA and SAHA have been shown to induce pancreatic cell death by apoptosis [60]. Other investigators have reported that TSA can synergize with gemcitabine [61] or the proteasome inhibitor PS-341 [62] to induce apoptosis of human pancreatic cancer cells. Thus, HDAC might also be promising targets for therapeutic intervention in pancreatic cancer.

With HDAC7 overexpression in pancreatic tumor tissues and not in their normal counterparts, it is reasonable to suggest that it might represent an interesting target for

novel approaches in the design of anti-pancreatic cancer therapies. However, rational targeting of HDAC7 signaling in this aggressive cancer disease needs further research to better understand its exact role in cancer progression. Also, it will require a clear understanding of cross-talk between HDAC7 signaling and other biochemical pathways that play a role in pancreatic cancer development. This may help to define which compound family could be more appropriate to be combined with HDAC7 inhibitors.

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Research Article

Influence of Hsp90 and HDAC Inhibition and Tubulin Acetylation on Perinuclear Protein Aggregation in Human Retinal Pigment Epithelial Cells

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Retinal pigment epithelial (RPE) cells are continually exposed to oxidative stress that contributes to protein misfolding, aggregation and functional abnormalities during aging. The protein aggregates formed at the cell periphery are delivered along the microtubulus network by dynein-dependent retrograde trafficking to a juxtannuclear location. We demonstrate that Hsp90 inhibition by geldanamycin can effectively suppress proteasome inhibitor, MG-132-induced protein aggregation in a way that is independent of HDAC inhibition or the tubulin acetylation levels in ARPE-19 cells. However, the tubulin acetylation and polymerization state affects the localization of the proteasome-inhibitor-induced aggregation. These findings open new perspectives for understanding the pathogenesis of protein aggregation in retinal cells and can be useful for the development of therapeutic treatments to prevent retinal cell deterioration.

1. Introduction

Retinal pigment epithelial (RPE) cells are exposed to chronic oxidative stress. They must constantly absorb light energy and phagocyte lipid rich photoreceptor outer segment shed from neural retina due to normal physiological visual cycle. Oxidative stress refers to progressive cellular damage that contributes to protein misfolding and functional abnormalities in the RPE cells during cellular senescence [1]. The accumulation of this damage in the postmitotic RPE cells seems to be one of the key events in the development of age-related macular degeneration (AMD), the leading cause of blindness in the elderly in the developed countries. The RPE cells ensure the survival of neural cells, rod, and cones. In senescent RPE cells, this ability is reduced causing secondary adverse effects on the neural retina, ultimately leading to loss

of vision. Both intra- and extracellular aggregation processes are crucial in cell degeneration and AMD [2].

Efficient removal of misfolded proteins from cytoplasm is critical for cellular survival and adaptation. However, potentially toxic misfolded protein aggregates accumulate during the aging process [3, 4]. Control of protein turnover is particularly important in postmitotic cells, since the accumulation of malfunctioning proteins may be highly detrimental to the cells [5]. Once heat shock protein-mediated protein folding fails, the misfolded proteins are usually tagged with a ubiquitin (Ub) moiety that directs the complex to the ubiquitin/proteasomal protein degradation pathway (UPP) [6]. It is believed that the aggregation of oxidized and ubiquinated proteins is due to a decline in the proteasomal activity with age, and that this also occurs in RPE cells [7–10]. Protein aggregates formed in the cell

periphery are delivered along microtubulus network by dynein-dependent retrograde trafficking to a juxtanuclear location where they form aggresomes [11–13]. The development of these aggresomes is part of a cellular defence mechanism against misfolded proteins [14], and it can be inhibited by drugs that depolymerize microtubules [15, 16].

Tubulin undergoes various posttranslational modifications including polyglutamylation, polyglycylation, carboxyterminal cleavage, and acetylation [17, 18]. Acetylation is unique among the known tubulin modifications, in that it occurs on the lysine 40 of α -tubulin which can be found on stable microtubules in most cell types [19]. The deacetylation of the lysine residue in tubulin is catalyzed by enzymes called histone deacetylases (HDACs). In contrast, histone acetyltransferases (HATs) transfer acetyl groups to lysine residue to increase acetylation of tubulin [20]. This results in a balance between acetylation and deacetylation states of tubulin, and any shift in this balance results in changes in the regulation of tubulin function [21]. The HDAC6 is the major cytoplasmic tubulin deacetylase [21–23]. Moreover, it efficiently binds mono- and polyubiquitin molecules [24–26]. One interesting role of tubulin and tubulin modifying deacetylases is their influence on aggregation of misfolded proteins [12]. HDAC6 contributes to the degradation of aggregated proteins because it is able to bind to both polyubiquitinated and dynein motor proteins as an adaptor protein to help transport misfolded proteins along microtubules into aggresomes which are finally degraded by autophagy [13, 22, 27–29]. HDAC6 is able to sense ubiquitinated cellular aggregates and consequently induces the expression of major cellular chaperones by triggering the dissociation of a repressive HDAC6/HSF1 (heat-shock factor 1)/HSP90 (heat-shock protein 90) complex and subsequent HSF1 activation. HDAC6 therefore appears as a master regulator of the cell protective response to cytotoxic protein aggregate formation [13].

We have recently shown that proteasome inhibition evoked perinuclear protein accumulation which then leads to the autophagy-mediated removal of the deposits [29]. In the present study, we demonstrate that Hsp90 inhibition with geldanamycin can effectively decrease the proteasome inhibitor-induced aggregation. Increased tubulin acetylation was observed in response to Hsp90, proteasome, and HDAC inhibition, but the acetylation level was not correlated with the amount of the aggregates in the ARPE-19 cells. However, the tubulin polymerization state did influence the localization of the aggregates.

2. Materials and Methods

2.1. Cell Culture and Treatments. Human retinal pigment epithelial cells (ARPE-19, from ATCC, [30]) were used in this study. The cells were grown in Dulbecco's MEM/Nut MIX F-12 (1:1) medium (Life Technologies, Paisley, UK) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 2 mM L-glutamine (Cambrex, Charles City, IA, USA), 100 units/ml penicillin (Cambrex), and 100 μ g/ml streptomycin (Cambrex). Before any exposure, the cells were grown to confluency in a standard incubator (10%

CO₂, +37°C). Proteasome inhibition was accomplished with 5 μ M MG-132 (Calbiochem, San Diego, CA, USA). Geldanamycin (GA, Calbiochem), at 0.25 μ M concentration, was used to inhibit the function of Hsp90. Microtubules were acetylated with 1 μ M trichostatin A (TSA; Sigma-Aldrich, Steinheim, Germany). Taxol (TAX, Paclitaxel; Calbiochem), at 1 μ M concentration and nocodazole (NOC; Calbiochem), at 5 μ M concentration, were used to disrupt to function of microtubules. The cells were exposed to the GA, TSA, TAX, or NOC for 24 h, simultaneously with MG-132 for 24 h or allowed to recover after 24 h MG-132 insult in medium with or without chemicals (GA, TSA, TAX or NOC).

2.2. Phase Contrast Microscopy. Phase contrast microscopy photographs were taken from live cells with a Nikon Eclipse TE300 (Nikon, Tokyo, Japan) microscope.

2.3. Electron Microscopy. For transmission electron microscopy (TEM), the cell culture samples were prefixed in 2.5% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.4) for 2 hrs at room temperature, followed by washing (15 min in 0.1 M phosphate buffer). The samples were postfixed in 1% osmium tetroxide (in 0.1 M phosphate buffer) for 1 hr at room temperature and washed as before prior to standard ethanol dehydration. Subsequently, the samples were infiltrated and embedded in LX-112 resin (Ladd Research Industries, Burlington, VT, USA). Polymerization was carried out at 37°C for 24 hrs and at 60°C for 48 hrs. The sections were examined in a JEOL-1200EX transmission electron microscope (Jeol, Tokyo, Japan) at 80 kV.

2.4. Western Blotting. Exposed cells were lysed in M-Per lysis buffer (Thermo Scientific, Waltham, MA, USA). Protein concentrations were analysed with the Bradford (Coomassie Brilliant Blue dye) method [31]. Whole cell extracts (20 μ g of protein) were run in 10% or 15% SDS-PAGE gels and then wet blotted to nitrocellulose membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The membranes were blocked for 1 hour in 3% fat-free dry milk in 0.3% Tween 20/PBS at room temperature (RT). Thereafter, the membranes were incubated for 1 hour at RT with rabbit polyclonal ubiquitin antibody (DakoCytomation, Glostrup, Denmark, cat. no. Z 0458) or rat monoclonal Hsp90 antibody (Assay Designs, Ann Arbor, MI, USA, cat. no. SPA-835) or mouse monoclonal Hsp70 antibody (Assay Designs, cat. no. SPA-810) or rat monoclonal Hsc70 antibody (Assay Designs, cat. no. SPA-815) or rabbit polyclonal LC3 antibody (Abgent, San Diego, CA, USA, cat. no. AP1802a) except for monoclonal acetylated tubulin antibody (Sigma-Aldrich, cat. no. T6793) where the incubation was for 30 minutes at RT. Primary antibodies were diluted (1:500, 1:5 000, 1:5 000, 1:5 000, 1:250 or 1:8 000 resp.) in 0.5% bovine serum albumin in 0.3% Tween 20/PBS except for acetylated tubulin which was diluted in 1% fat-free dry milk in 0.05% Tween 20/PBS. After 3 \times 5 minutes washes with 0.3% Tween 20/PBS (for acetylated tubulin 0.05% Tween 20/PBS) the membranes were incubated for one hour at RT with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG antibodies or anti-rat IgG antibodies (GE Healthcare).

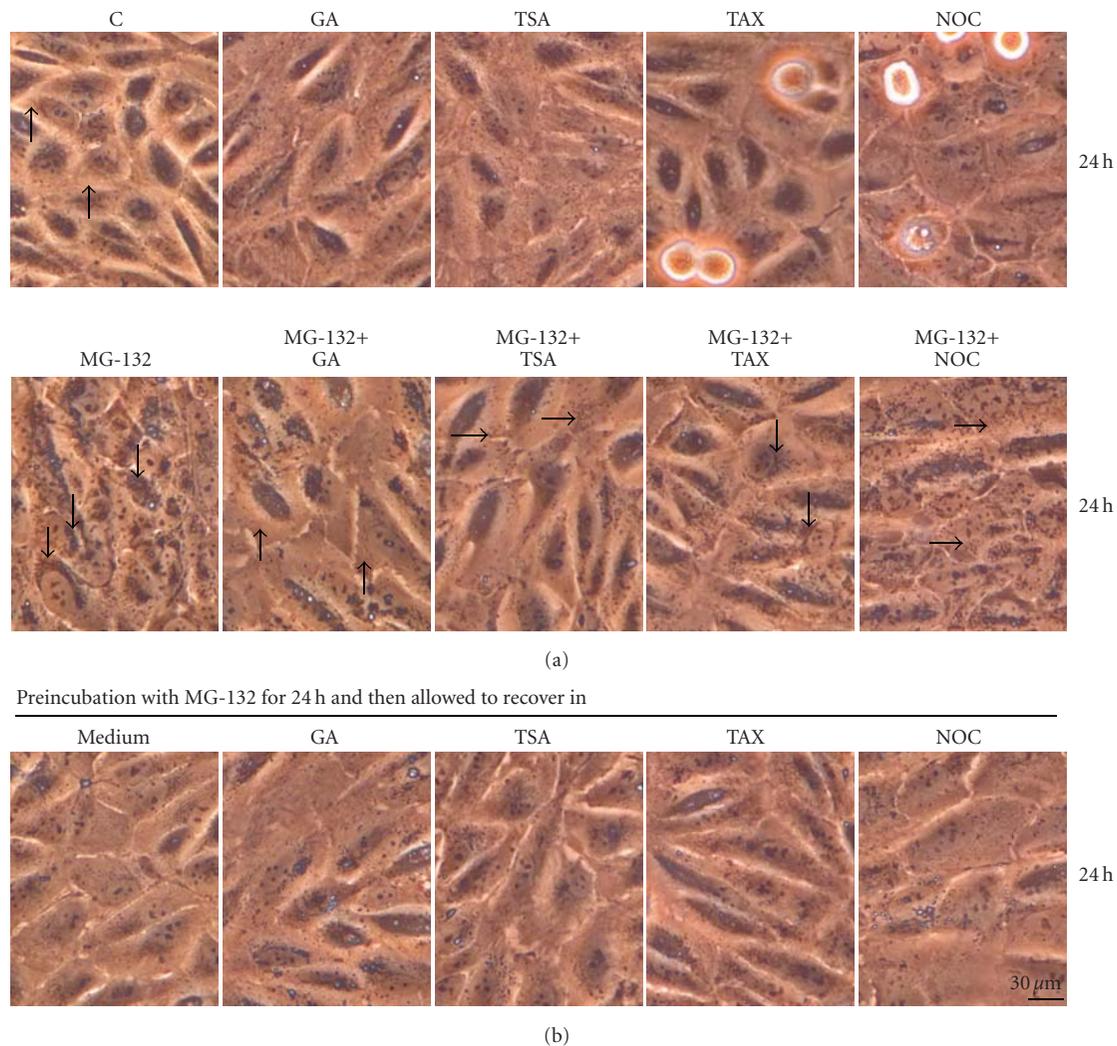


FIGURE 1: (a) Phase contrast micrographs of control (C) cells, and cells exposed to 5 μM MG-132 (MG) or 0.25 μM geldanamycin (GA) or 1 μM trichostatin A (TSA) or 1 μM taxol (TAX) or 5 μM nocodazole (NOC) for 24 hours. In addition, the cells were treated simultaneously with GA or TSA or TAX or NOC and MG-132 for up to 24 hours, (b) and then allowed to recover under the indicated conditions for 24 hours. Protein aggregates are seen as dark granular deposits in perinuclear space (downward arrows), the examples of clear cytoplasm are marked with upward arrows. Arrows from left to right indicate disperse aggregation.

The secondary antibodies were diluted (for ubiquitin 1:20 000, for Hsp90 1:10 000, for Hsp70 1:40 000, for Hsc70 1:15 000 and for LC3 1:5000, resp.) in 3% fat-free dry milk in 0.3% Tween 20/PBS except for acetylated tubulin (1:6 000) which was diluted in 1% fat-free dry milk in 0.05% Tween 20/PBS. Before detection, all of the membranes were washed as before. Protein antibody complexes were detected with an enhanced chemiluminescence method (Millipore, Billerica, MA, USA). The western blots were quantified with Quantity One software 4.5.0. (Bio-Rad, Hercules, CA, USA).

3. Results and Discussion

3.1. Hsps and Protein Aggregation. The ARPE-19 cells were either nonstressed, or exposed to drugs, that is, 5 μM MG-132 or 0.25 μM geldanamycin, 1 μM trichostatin A or 1 μM taxol or 5 μM nocodazole for 24 hours. In addition, the cells

were treated simultaneously with MG-132 in conjunction with geldanamycin, trichostatin A, taxol or nocodazole up to 24 hours. Only a single MG-132 insult or combination treatment with taxol induced a typical perinuclear aggregation in ARPE cells (Figures 1(a) and 2(a)). Interestingly, Hsp90 inhibition with geldanamycin effectively suppressed MG-132 induced protein aggregation. HDAC inhibition with trichostatin A or tubulin depolymerization with nocodazole evoked dispersed the mid-peripheral aggregation process during proteasome inhibition. With all of the insults, cytoplasm underwent a similar effective aggregation clearance when exposed to MG-132 for 24 hours and then allowed to recover for 24 hours in normal cell culture medium (Figures 1(b) and 2(b)).

A robust elevation of Hsp70 protein expression was seen in response to MG-132 or geldanamycin exposures (Figure 3). Hsp90 levels were not markedly affected by the

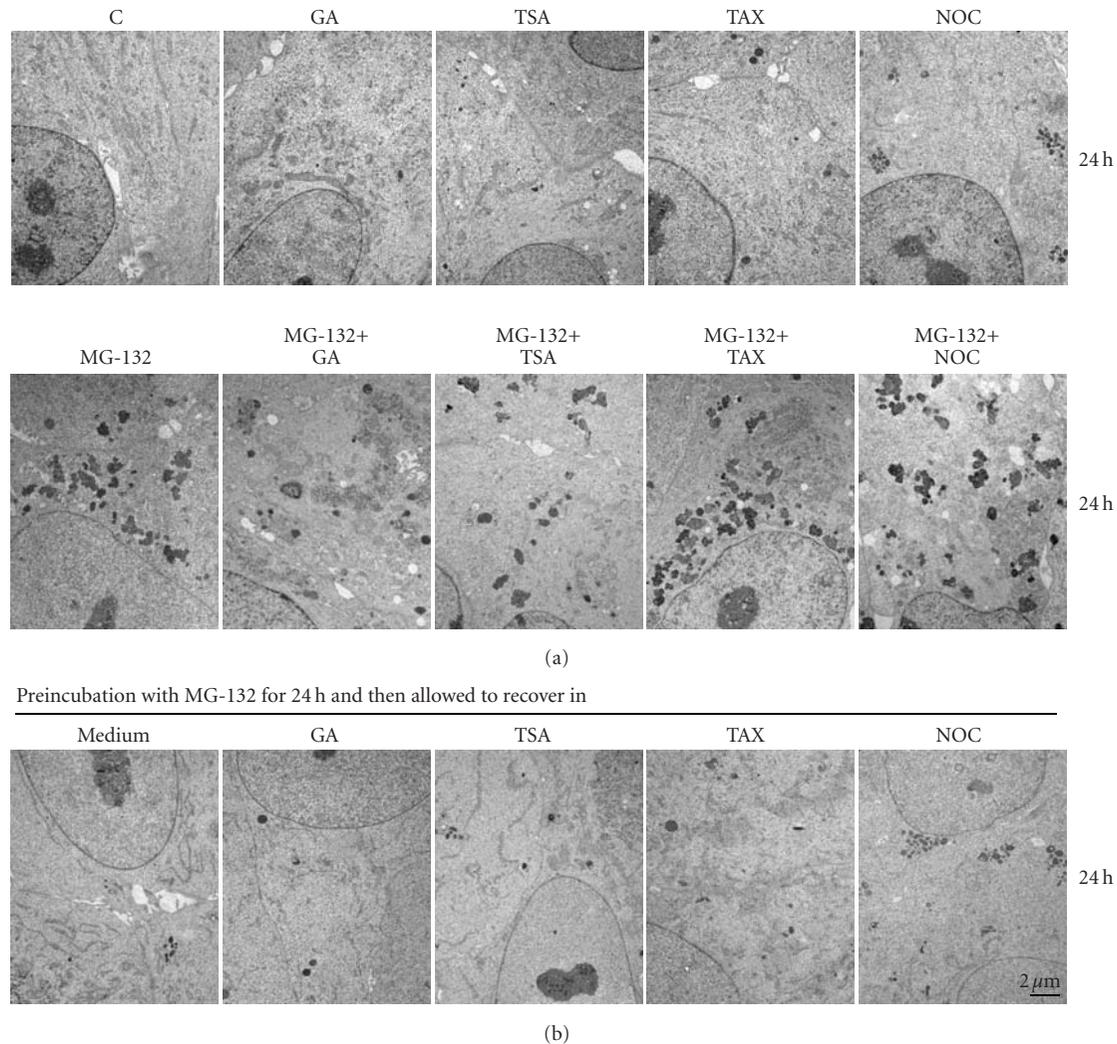


FIGURE 2: (a) Transmission electron micrographs of control (C) cells, and cells exposed to 5 μM MG-132 (MG) or 0.25 μM geldanamycin (GA) or 1 μM trichostatin A (TSA) or 1 μM taxol (TAX) or 5 μM nocodazole (NOC) for 24 hours. In addition, the cells were treated simultaneously with GA or TSA or TAX or NOC and MG-132 for up to 24 hours, (b) and then allowed to recover under the indicated conditions for 24 hours.

treatments. Abrogation of proteasome-mediated protein degradation caused a clear increase in the amount of Hsp70 which has a cytoprotective capacity in the MG-132-treated ARPE-19 cells [29]. The classical transcriptional heat-shock gene induction is attributable to the activation of HSF1 transcription factor [32]. In the activation process Hsp90 and Hsp70 dissociate from HSF1 transcription factor [33, 34]. Geldanamycin has been shown to bind Hsp90, to inhibit its function and to elicit the Hsp90 client protein degradation in proteasomes [35, 36]. In line with previous studies [37–39], the geldanamycin was found to trigger a strong expression of Hsp70, while the Hsp90 response remained weaker. The response is likely mediated through HSF1 transcriptional activation [33, 34]. The increase in the amount of inducible Hsp70 might be one of the regulators suppressing the proteasome inhibitor-induced aggregation process [29], when Hsp90 is simultaneously inhibited. It has also been documented that a Hsp90 inhibitor may prevent

the aggregation of protein by regulating client protein posttranslational modifications [40]. Since Hsp90 inhibition has been reported to trigger autophagy clearance [41, 42], we wished to analyze the autophagy induction marker LC3 I/II levels in ARPE cells treated with geldanamycin or MG-132 solely or both together for 6, 12, and 24 hours. Our findings clearly show that proteasome inhibition mildly induced autophagy, when related to LC3 II levels, but this was not involved in geldanamycin treatments (Figure 4). This indicates that autophagy clearance is not implicated in the suppression of protein aggregation during Hsp90 and proteasome inhibition in the ARPE-19 cells.

3.2. Ubiquitination. Cellular ubiquitin-protein (Ub) conjugate levels were analyzed by western blotting from cells treated as described above. The level of Ub conjugation was not changed by geldanamycin, trichostatin A, taxol, or nocodazole treatment, but a very intensive induction of

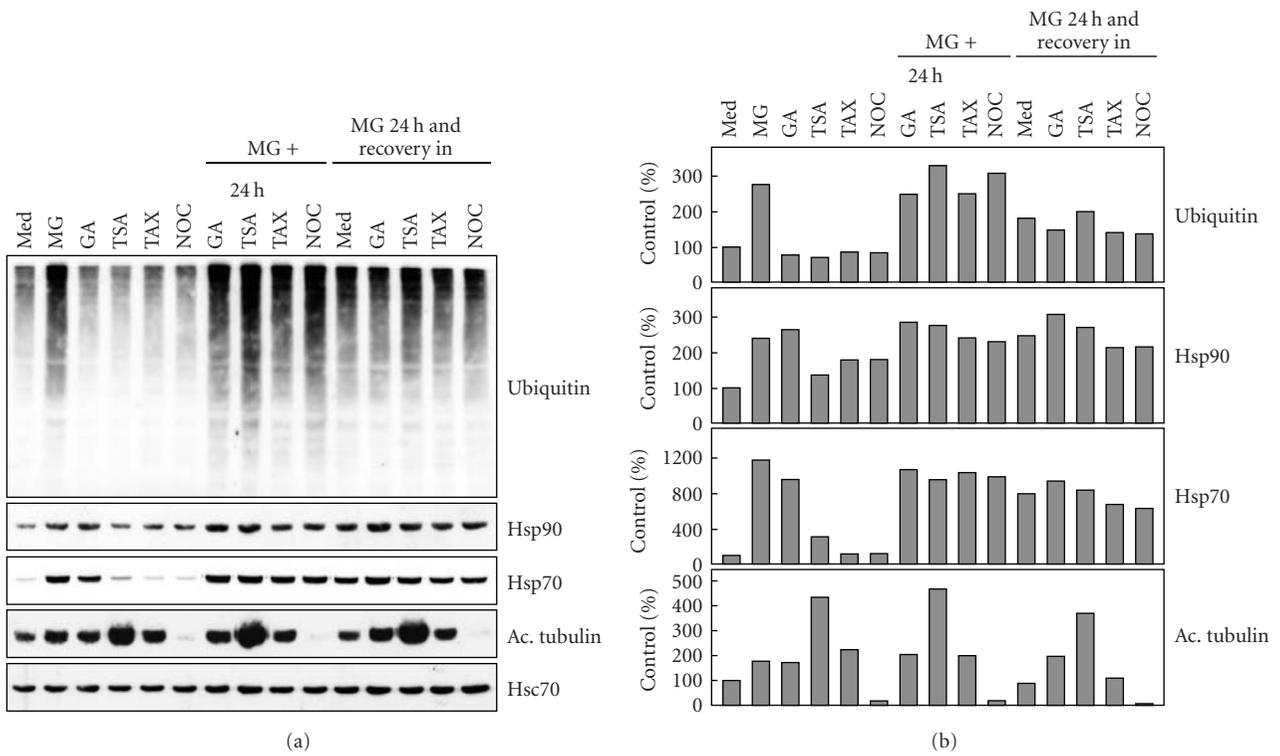


FIGURE 3: (a) Total proteins (20 μ g) of whole cell extracts were examined by western blot using antibodies against ubiquitin, Hsp90, Hsp70, acetylated tubulin, and Hsc70 of control (C) cells, and cells exposed to 5 μ M MG-132 (MG) or 0.25 μ M geldanamycin (GA) or 1 μ M trichostatin A (TSA) or 1 μ M taxol (TAX) or 5 μ M nocodazole (NOC) for 24 hours. In addition, the cells were treated simultaneously with GA or TSA or TAX or NOC and MG-132 for up to 24 hours and then allowed to recover under the indicated conditions for 24 hours. Hsc70 was used to check the equal loading of proteins. (b) Quantifications of western blots.

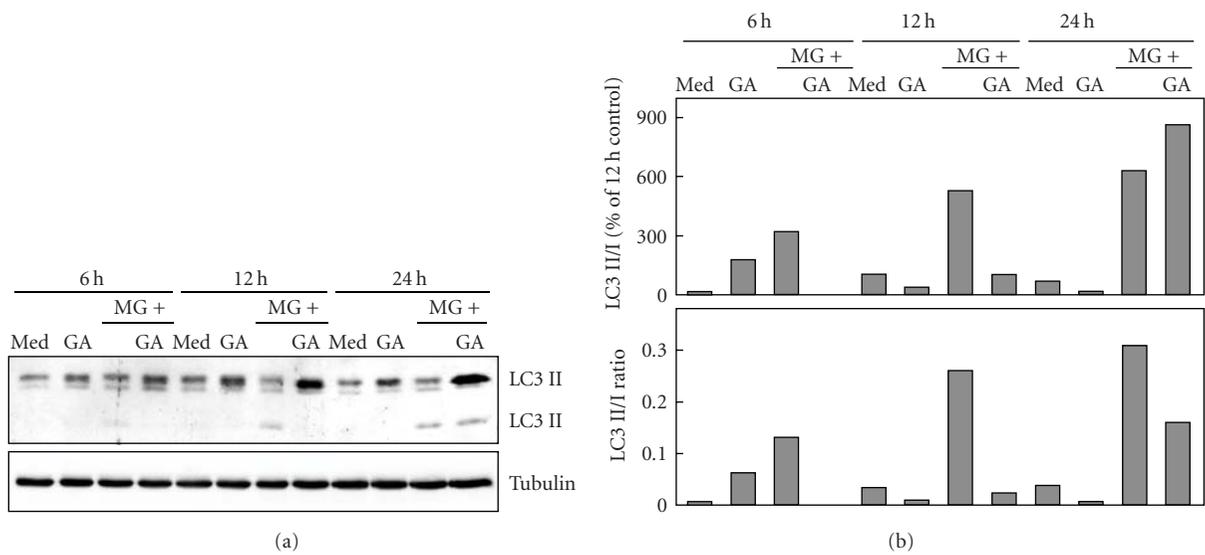


FIGURE 4: (a) Total proteins (20 μ g) of whole cell extracts were examined by western blot using antibodies against LC3 of control (med) cells, and cells exposed 0.25 μ M geldanamycin (GA) or 5 μ M MG-132 (MG) or their combination for 6, 12, and 24 hours. Tubulin was used to check the equal loading of proteins. (b) Quantifications of western blots and LC3 II/I ratio.

ubiquitination was seen in response to MG-132 exposure whether geldanamycin, trichostatin A, taxol, or nocodazole were present or not (Figure 3). Inhibition of Hsp90 therefore seems to have only a minor impact on the level of ubiquitination in the ARPE-19 cells, although Hsp90 has been shown to regulate both proteasomal and autophagy clearance through its client proteins [41–43]. The ubiquitin levels were slightly reduced when proteasome inhibition was removed and the cells recovered either with geldanamycin, trichostatin A, taxol, or nocodazole compared to incubations of MG-132 together with these chemicals. This is apparently explained by the autophagic clearance of proteasome-inhibitor-induced aggregates, as we have recently documented [29].

3.3. Acetylation. Protein aggregates formed at the cell periphery are delivered along the microtubulus network by dynein-dependent retrograde trafficking to a juxtanuclear location where they form aggresomes [11, 12]. The reversible acetylation of α -tubulin has been linked to the regulation of microtubule stability and function [21]. Decreased tubulin acetylation has been shown in some reports to reduce microtubule stability [21, 22], but in other experiments no association was observed [44, 45]. HDAC6 deacetylates tubulin, and Hsp90, and forms complexes with many other proteins, including ubiquitinated proteins. HDAC6 also interacts with a component in the dynein-dynactin microtubule motor complex and regulates protein aggregation trafficking [13, 45, 46]. Trichostatin A is a classical inhibitor of HDAC deacetylase activity and thus it also blocks the HDAC6 [47]. The phase contrast and transmission electron microscopic analysis revealed dispersed cytoplasmic aggregation in response to simultaneous treatment of trichostatin A or nocodazole and proteasome inhibition (Figures 1 and 2). Trichostatin A evoked a clear increase in tubulin acetylation levels, while in the situation of tubulin depolymerizator induced by nocodazole, tubulin acetylation remained at control level (Figure 3). In contrast, a tubulin stabilizer, taxol induced tubulin acetylation that was not, however, as extensive as that evoked by trichostatin A. Hsp90 and proteasome inhibition evoked a similar slight increase in the tubulin acetylation levels. All these findings indicate that total tubulin acetylation is not related to perinuclear aggregation. The HDAC inhibition by trichostatin A probably affects the dynein motor and regulates aggregation localization, but does not prevent the formation of aggregates. Moreover, the polymerization state of tubulin regulates the localization of proteasome inhibitor-induced aggregates, but this process cannot be estimated by measuring total tubulin acetylation levels. Hsp90 inhibition seems to prevent aggregation rather than regulating trafficking through the tubulin network.

4. Conclusions

Hsp90 inhibition is effectively involved in the regulation of protein aggregation that is independent of HDAC inhibition or tubulin acetylation levels in the RPE cells. These findings open new perspectives for understanding the pathogenesis of protein aggregation in retinal cells, and they may be useful in

the development of therapeutic treatments to prevent retinal cell deterioration, that is, during aging.

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