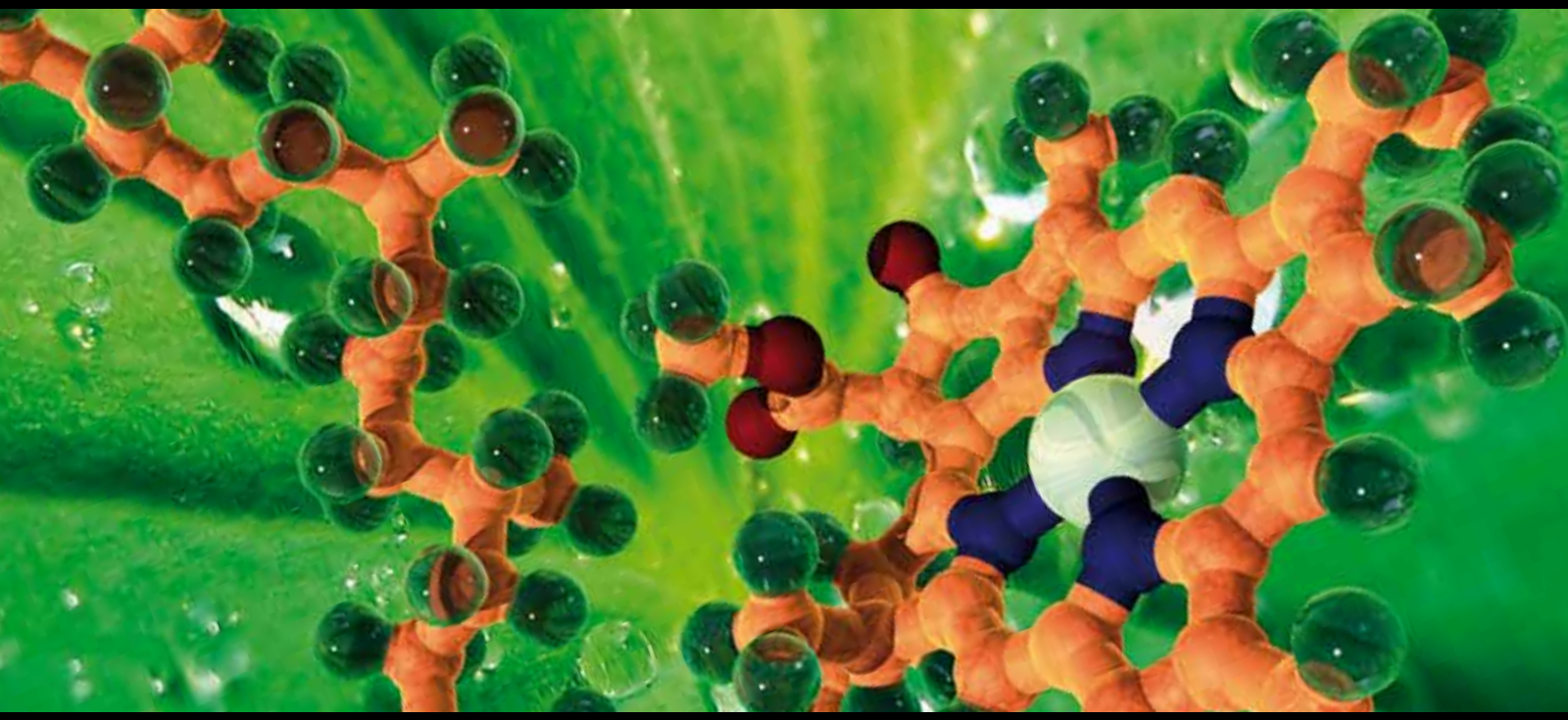
Biochemistry Research International

Ubiquitin Proteasome System in Stress and Disease

Guest Editors: Dmitry Karpov, Michael H. Glickman, Shoshana Bar-Nun, and Philip Coffino



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Editorial Board

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Editorial

Ubiquitin Proteasome System in Stress and Disease

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The proteasome is an ATP-dependent multisubunit self-compartmentalised protease complex. It is the major regulator of intracellular proteins turnover. The majority of proteins degraded by proteasomes are modified by covalent attachment of polyubiquitin chains. Substrates include regulatory proteins, transcription factors, components of signal transduction pathways, and proteins that have aberrant structures. The ubiquitin-proteasome system (UPS) thus participates in regulating diverse and numerous cellular processes. Identifying novel proteasome substrates and understanding the mechanisms of proteasome-dependent protein degradation require careful planning and implementation of methods for measuring the rate of protein degradation. Beatriz Alvarez-Castelao et al. discuss state-of-the-art methodologies for investigation of protein degradation. They focus on sources of potential experimental pitfalls and suggest ways to avoid them.

Apart from ubiquitin, there is a large family of ubiquitin-like protein modifiers that are also involved in multiple forms of proteolysis-dependent and proteolysis-independent regulation. The SUMO (small ubiquitin-like modifiers) family is a well-studied example. Investigation of mechanisms of SUMO-dependent processes requires identification of the sites of SUMOylation and/or SUMO-interacting proteins and sequences. Elisa Da Silva-Ferrada et al. present a survey of the current methodologies used to study SUMO-regulated functions and identification of *cis* and *trans* sequences controlling SUMOylation. Based on critical consideration of previously described methods, they provide guidelines for selection of the appropriate method depending on the research goal.

If polyubiquitination mainly serves for proteasome-dependent protein degradation, protein monoubiquitination may be read as a signal of proteasome-independent regulation of protein activity. A well-known example is the internalization and endosome sorting of membrane receptors. Michel Becuwe et al. describe mechanisms of action of arrestins, a highly conserved protein family and one of the key regulators of G-protein coupled receptors (GPCR) signaling. Arrestins may regulate GPCR activity by serving as ubiquitin-ligase adaptors. It should be noted that Robert J. Lefkowitz and Brian K. Kobilka were awarded the 2012 Nobel Prize in Chemistry for their pioneering studies of GPCR. Interestingly, arrestins themselves are regulated by ubiquitination/deubiquitination. A recently found family of arrestin-like proteins is involved in regulating the activity of membrane transporters and other membrane-bound proteins. For example, they can participate in proteolytic activation of membrane-bound precursors of transcription factors.

The UPS system has its own quality control of substrate ubiquitination. This function is performed by a class of deubiquitinating enzymes. Misregulation of deubiquitination may be a significant factor in cancer progression. Jennifer Hurst-Kennedy et al. have reviewed the current status of our knowledge on the role of an unusual deubiquitinating enzyme, ubiquitin C-terminal hydrolase L1 (UCH-L1), in tumorigenesis. Normally, UCH-L1 is an abundant neuronal

protein but its expression level is also increased in malignancies. The authors suggest that UCH-L1 may serve as an early biomarker of malignancy as well as a potential therapeutic target.

UPS dysfunction may contribute to human disorders, and among them are severe neurodegenerative diseases. Sabine Schipper-Krom et al. describe the UPS involvement in progression of Huntington's disease. The N-terminal fragments of huntingtin (N-htt), which contain polyQ repeats, are prone to form aggregates and serve as the sources for toxic polyQ oligopeptides. This review focuses on two controversial issues, the impairment of UPS sequestered into htt aggregates, and the degradation by the proteasome of polyQ-containing proteins. The authors conclude that N-htt does not directly affect proteasome activity and proteasomes are not irreversibly sequestered into htt aggregates. Possible therapeutic means to augment proteasome activity against polyQ-containing proteins are suggested.

These review papers represent an exciting, insightful observation into the state-of-the-art, as well as emerging future topics. We hope that this special issue would attract a large attention of the peers and inspires them to conduct fruitful experiments. We would like to express our appreciation to all the authors and reviewers for their great support that made this special issue possible.

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Review Article

A Critical Appraisal of Quantitative Studies of Protein Degradation in the Framework of Cellular Proteostasis

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Protein homeostasis, proteostasis, is essential to understand cell function. Protein degradation is a crucial component of the proteostatic mechanisms of the cell. Experiments on protein degradation are nowadays present in many investigations in the field of molecular and cell biology. In the present paper, we focus on the different experimental approaches to study protein degradation and present a critical appraisal of the results derived from steady-state and kinetic experiments using detection of unlabelled and labelled protein methodologies with a proteostatic perspective. This perspective allows pinpointing the limitations in interpretation of results and the need of further experiments and/or controls to establish "definitive evidence" for the role of protein degradation in the proteostasis of a given protein or the entire proteome. We also provide a spreadsheet for simple calculations of mRNA and protein decays for mimicking different experimental conditions and a checklist for the analysis of experiments dealing with protein degradation studies that may be useful for researchers interested in the area of protein turnover.

1. Introduction: Cellular Proteostasis

The living cell requires a homeostatic control of energy, use, and production to accomplish the different cell functions. Proteins are the main producers, users and transformers of energy. The set of proteins that are present in a cell at a given time is what we call the cell proteome. The cellular proteome has to take care of itself and its behaviour determines cell function. Accordingly, the proteome has its own homeostasis that is necessarily coupled, at least, to energy homeostasis. Protein homeostasis, proteostasis, is critical for the adaptation of cell function to a fluctuating internal and external milieu. Those adaptative responses, like regular exercise for us, keep the proteome in good "shape." The proteostatic mechanisms of a cell involve a complex network of pathways that includes protein synthesis, folding, posttranslational modifications (PTMs), protein-protein interactions (PPIs), subcellular localization, and degradation. Protein expression levels in eukaryotes are determined by several processes, beginning with nuclear gene expression. Nuclear gene transcription, pre-mRNA processing, mRNA nuclear transport, and degradation (Figure 1, BOX 1) are the initial steps determining the available pool of cell mRNAs that can be translated, the translatome, the total mRNAs that are in ribosome complexes undergoing translation (Figure 1, BOX 2). The life of a protein begins as a nascent polypeptide by translation of its mRNA (Figure 1, BOX 2). The "survival" or "demise" of the nascent polypeptides and the newly synthesized proteins is under control. Many cellular proteins can be degraded at this early stage of its biogenesis, including those that are defective that constitutes the so-called defective ribosomal products (DRiPs) and that could potentially account for up to 30% of the polypeptides synthesized by a mammalian cell [1]. In this early stage, correct folding of the newly synthesized proteins to its functional tertiary and quaternary structures (Figure 1 BOX 3) is assisted by dedicated chaperones that also play an important role in reverting misfolding [2]. Specific PTMs and PPIs of the nascent, newly

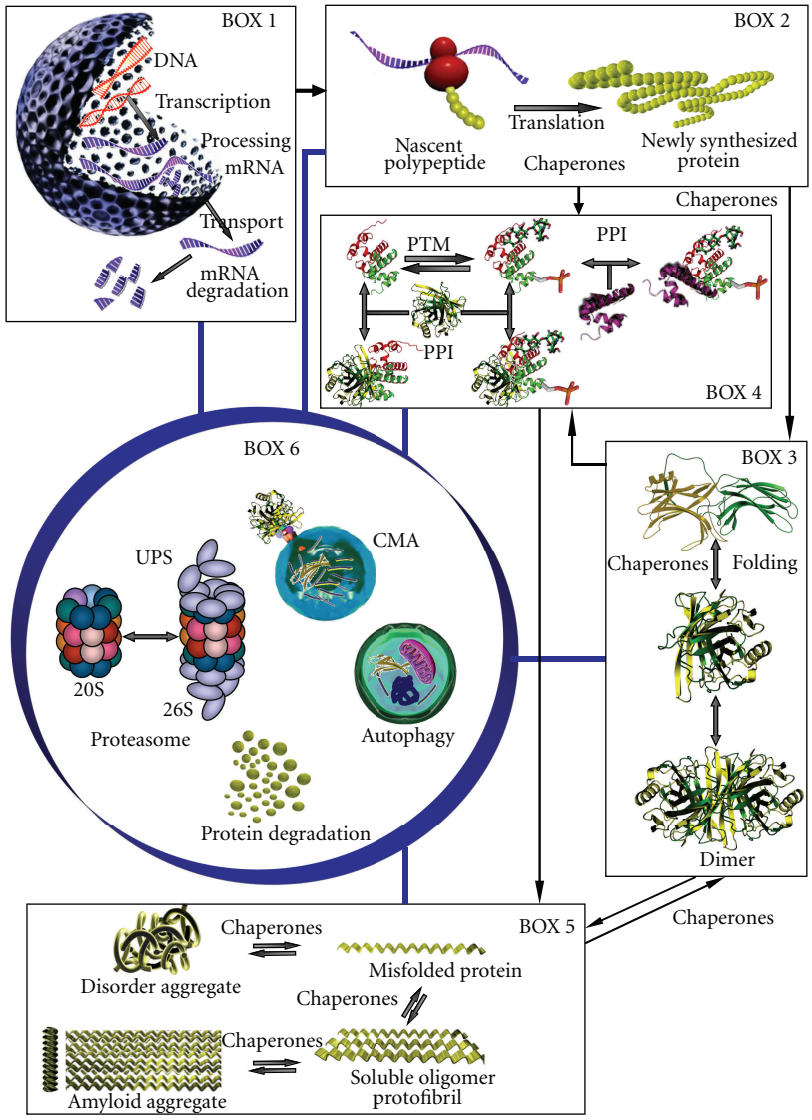

Figure 1: Schematic diagram of cell proteostasis. The boxes illustrate the different cellular process involved in protein homeostasis. BOX 1, nucleus, where gene transcription and pre-mRNA processing produce the mature mRNA that will be transported to the cytoplasm where it could be degraded (mRNA decay can also take place in the nucleus). The mRNA is engaged, mainly in the cytoplasm, to translation by the ribosomal machinery producing a nascent polypeptide that grows to a newly synthesized protein (BOX 2). The folding of the newly synthesized proteins, helped by chaperones, results in the "so-called" native protein structure either monomeric or oligomeric (BOX 3, only a dimer is shown for simplicity). Both the newly synthesized proteins and the mature mono or oligomeric forms of proteins are subjected to post-translational modification (PTM) and specific and unspecific protein-protein interactions (PPIs) that are illustrated in BOX 4. Proteins due to changes in its native conformation produced by different physical and/or chemical perturbations of the cell, or by mutations, could get misfolded or misprocessed and misfolded. The misfolded proteins, perhaps under the influence of PPIs and PTMs, will produce protein aggregates or soluble oligomeric protofibrils that eventually may form amyloid fibers (BOX 5). The degradation of proteins (BOX 6) is mainly due to the ubiquitin-proteasome System (UPS, nucleus and cytoplasm, 20S, and 26S proteasome) and the autophagic pathways (cytoplasmic): mainly chaperone-mediated authopagy (CMA) and macroautophagy (autophagy). Other proteases also participate in protein degradation (calpains, caspases, etc.), not shown. Blue lines connect all the boxes to the central circular box of protein degradation (BOX 6) indicating that proteolysis can regulate any of the process and vice versa. Black arrows connecting boxes indicate the "flow" of the products depicted in each BOX.

synthesized or the mature native proteins are due to their living circumstances in a general crowded environment controlled by thermal motion and diffusion rates (Figure 1, BOX 4) with an estimated protein concentration close to 30 mM [3]. Due to physical and/or chemical modification, native proteins may get misfolded given rise to the formation of aggregates or protofibrils which eventually organized into amyloid fibers (BOX 5). The ubiquitin proteasome and autophagic pathways (Figure 1, BOX 6) are the main pathways of general protein degradation. PTMs and PPIs together

with subcellular localization determine protein degradation by those pathways. The subcellular localization of proteins certainly contributes to their proteostasis: protein trafficking, folding, and regulation of degradation of the specific translatome for mitochondria (chloroplasts), the endoplasmic reticulum, and the secretary pathway; the peroxisomes and the cell nucleus have mechanistic differences from the cytoplasmic protein proteostasis [4–8]. In addition, the localization of mRNAs and the translational machinery in the cell are also highly relevant to proteostasis. A special case is neurons that have specialized compartmentalization, dendrites, and axons. Transport of some mRNAs and the translational machinery to those distant places from the neuronal cell soma and the retrograde transport to the soma are crucial step to maintain proteostasis at dendrite and axons, but they may also exist proteostatic mechanisms at those locations likely to be both quantitatively and qualitatively different from soma proteostasis [9, 10]. Finally, cellular proteostasis may be controlled by signalling pathways in a cell-nonautonomous manner that remain to be identified [11].

The energetic cost of the production and maintenance of a healthy proteome is critical to determine life sustainability and is probably one of the reasons why protein and energy homeostasis have coevolved to allow the appearance of complexity. Accordingly, their regulatory mechanisms have important crosstalks and are well conserved during evolution [12]. Energy and/or protein homeostasis failure leads to cell dysfunction and eventually to cell death, as may occur during aging, neurodegenerative diseases [13], and possibly in other chronic human diseases; diabetes, cancer, renal and pulmonary fibrosis, and so forth. Certain proteins, like toxic metabolites, can become proteotoxic and the cell has also mechanisms to cope with those toxic proteins (Figure 1, BOX 5), in particular for misfolded or aggregated proteins [14]. The global connection of energy and protein homeostasis is also supported by recent experiments showing that significant increase of life span results from reduction of insulin/IGF signaling or by loss of VHL/HIF1, that also improves function of transgenic models of proteotoxic diseases as reported for *C. elegans* [15, 16] and mice [17].

Studies on protein turn-over are used by many researchers in many different areas of cellular and molecular biology, but sometimes the interpretations of the results are leading to incorrect conclusions. This paper deals with the experimental approaches used to study protein degradation in a quantitative basis and how a critical appraisal of the interpretations of the results in the framework of proteostasis would certainly help to arrive to appropriate conclusions, knowing what we can reasonably conclude from each experimental setup.

2. Steady-State versus Kinetic Studies

By definition, the steady-state level of each protein of a cell proteome is attained when its rate of synthesis and its rate of degradation are equal, invariant respect to time. Also by definition, except in the quiescent state, cells will grow and divide generating two daughter cells; as a consequence, the entire proteome is diluted by the increase in cell volume due to cell division cycle. Each protein of the proteome will have its own steady-state levels, and changes in the steady-levels are the result of changes in the rate of protein synthesis and/or the rate of protein degradation.

The rate of synthesis of the entire proteome of a cell is controlled by the relative abundance of the different mRNAs of the transcriptome, whose different steady-state levels are also the result of mRNA synthesis and degradation, and by their translational rate. The actual amount of a protein being synthesized is the product of the copy number of mRNA molecules and the number of protein molecules synthesized by unit time per mRNA molecule. The rate of degradation of mRNAs or proteins is generally assumed to follow an exponential decay, similar to the familiar radioactive decay of an isotope. Accordingly, the changes over time of the amount of a cell protein can be expressed with the following differential equation, taken from Belle et al. [18],

$$P(t) = \frac{dP}{dt} = M(t) * R - P(t) * (D + V), \tag{1}$$

where P is the protein concentration, M is the copy number of the mRNA, R is the rate of translation per mRNA molecule, D is the protein degradation rate constant, and V is the growth rate (volume increase factor per unit time). At steady state, the rate of protein synthesis and degradation are equal, and as a consequence, $dP/dt = 0$, no change in the amount of protein over time. The steady-state levels of a protein can be defined by the following equation, also taken from Belle et al. [18],

$$(P0) = \frac{M(0) * R}{D + V}. \tag{2}$$

We provide an on-line spreadsheet that allows for easy graphical visualization of the rate of protein degradation after the introduction of different values for the terms of the differential equation: number of RNA molecules, rate of mRNA translation, mRNA half-life, protein half-life, and cell doubling times.

Nowadays, the heavy/light isotope pulse-labelling approach, stable isotope labeling by amino acids in cell culture (SILAC), followed by mass spectrometry- (MS-) based quantitative proteomics, is the method of choice to measure protein turn-over rates under steady-state conditions [19, 20] and in a proteomic scale. The SILAC/MS methodologies when possible, and others like the isobaric tag for relative and absolute quantification (iTRAQ), are presently used to measure degradation rates (half-lives) of proteins. These methodologies can also be used to compare relative protein abundance in cells that have been subjected to a stimulus, moving from one to another steady state and even for single cell analysis [21]. New technical developments are being designed that may allow the inclusion of the study of PTMs effects on protein turn-over changes in response to a cell stimulus or stress [22]. Nevertheless, more "traditional" methods to measure protein turn-over are still widely used. There are two basic experimental designs: measuring the amount of a certain protein before and very long after a cell

perturbation and measuring the changes in protein concentrations by kinetic, time-course experiments. In the first approach, enough time is provided after cell perturbation to ensure that the cell moves from the initial to a new steady state. In this steady-state situation, the observed changes in protein levels can be attributed to either changes in protein synthesis or degradation, or to both, changes in synthesis and degradation. In the kinetic approach, time-course experiments, the contribution of each of the branches (synthesis and degradation) to the new steady state after cell perturbation is studied. The analysis of these approaches will be the focus of the following sections of this paper.

3. Pharmacological and Genetic Interference Studies of Protein Degradation

The most common experimental setup to study protein degradation is to measure changes in protein levels mainly by protein immunoblot of total cell extracts. The pharmacological interference to study protein degradation is performed by treatment of cells with protein synthesis and/or degradation inhibitors. Genetic interference to study protein degradation targets either transcription or mRNA function. Transcriptional downregulation of mRNA expression is achieved by the use of Tet-on or Tet-off transcriptional control of the corresponding gene. The function of mRNA is targeted by specific siRNA or shRNA that results in RNA decay and/or inhibition of protein synthesis of the targeted mRNA.

3.1. Pharmacological Interference Studies of Protein Degradation. One of the simplest experiments is to add an inhibitor of protein degradation to the cells, wait overnight or 24 h, and then compare the levels of the protein of interest between the untreated and the treated cells by simple immunoblot of total cell extracts. When the results obtained show an increase in the protein levels, the usual conclusion is that the protein is being degraded by the degradation pathway that is "specifically" blocked by the inhibitor. When there is no change or a decrease in the protein levels, the particular pathway of degradation blocked by the specific inhibitor does not participate in the degradation of the protein. Those conclusions seem appropriate, but can be incorrect. Most of those experiments are performed after prolonged incubation times (as mentioned above), and as a consequence, they are measuring changes in the "steady-state" levels of a protein. The new steady-state level of a protein after treatment with an inhibitor of protein degradation can be reached kinetically by changes in its degradation rate with a constant rate of synthesis, by changes in its rate of synthesis, or by both changes in synthesis and degradation. As a consequence, in those experiments further experiments are needed, before reaching a conclusion. Those controls should include the study of the possible effects of inhibitors of protein degradation on the rate of synthesis of the protein under study. Specifically to measure changes in the transcription (or decay) of the corresponding mRNA that may result in an increase (decrease or no change) in the number of RNA molecules being translated or even the actual rate of mRNA translation in the presence and in the absence of the protein degradation inhibitor.

The aforementioned situation is very clearly exemplified by the broadly used proteasome inhibitors. Those inhibitors are "very specific," but the ubiquitin-proteasome degradation pathway is also implicated in transcriptional control. The ubiquitin-proteasome pathway participates in the degradation of transcription factors limiting the transcriptional output, and also in recycling of transcriptional complexes on chromatin to facilitate multiple rounds of transcription [23–25] affecting the amounts of mRNA available for translation. As a consequence, if the gene encoding the protein whose degradation is being studied is controlled at the transcriptional or posttranscriptional levels (mRNA processing, nuclear export, mRNA decay, or translation) by the ubiquitin-proteasome pathway, part of the change in steady-state protein levels could be explained by modulation of those upstream mechanisms (Figure 1, affected processes depicted in BOXES 1 and 2). Those upstream processes become especially relevant for proteins with a short half-life (minutes to a few hours). We have shown that transcriptional upregulation of the CMV promoter is the major cause of the increased protein levels of unstable fluorescent reporter proteins after treatment of cells with proteasome inhibitors, and not as much to its decreased rate of degradation by inhibition of the proteasome [26]. The steady-state levels of the unstable fluorescent proteins under basal conditions are very low for a given rate of synthesis and degradation (steady state), the increase in the amounts of the unstable protein by treatment with proteasome inhibitors cannot be accounted by the same rate of protein synthesis as in the basal conditions and simple inhibition of the degradation of the unstable protein. In fact, the increase in protein levels of unstable fluorescent proteins in the presence of proteasome inhibitors can be prevented by cotreatment of the cells with transcriptional inhibitors [26]. The same caveat also applies for proteins with longer half-lives, but in this case the effect observed may be masked due to the higher abundance of those proteins under steady-state conditions that makes more difficult to observe significant changes by immunoblotting.

A similar situation applies to the interpretation of the effect of proteasome inhibitors in time-course experiments. As an example from published results, let us analyze recent papers on the effect of proteasome inhibitors on the ubiquitinome (total ubiquitylated proteins of a cell) protein landscape. Two recent studies [27, 28] state that changes in the abundance of the ubiquitinome are not reflective of overt protein accumulation in response to addition of proteasome inhibitors to the cells. This statement does not mean that there are no clear changes in the abundance of different proteins. In the time-course study of HCT116 cells treated with 1 μM bortezomib reported by Kim et al. [27], the average change in protein accumulation is 1.5-fold after 8 h of treatment (for more than 4500 and less than 5744 protein being analyzed). The corresponding average increase for ubiquitylated modified peptides is 2.8-fold. As the authors already point out that they could not analyze many known low-abundance canonical ubiquitin-proteasome targets, their

conclusion should be taken with caution. Further cautions apply to those studies; do not get blinded by high numbers and the use of log2 scales for expressing the results. An average increase of 1.5-fold in the total abundance of the "proteome" after treatment of cells with proteasome inhibitors for 8 h is a tremendous energetic burden for the cell proteostasis, mainly energy expenditure for protein synthesis and folding, and a small fraction of this accumulation could be explained by inhibition of protein degradation (see below). These results clearly show the potent effect of proteasome inhibitors on global regulation of gene expression, but the details are also interesting. For example, the key enzyme controlling glycolysis, phosphofructokinase (PFK), has a half-life of 40–70 h (depending on the isozyme) as recently determined by a SILAC-MS proteomic studies in HeLa and C2C12 cells [29], and is not expected to be very different in HCT116. Accordingly, treatment of cells with 1 μM bortezomib for up to 8 h should not change significantly the total amount of PFK in the cell by inhibition of its degradation. In contrast, Kim et al. [27] report that the abundance of PFK isozymes increase threefold (1.5 on log2 scale used by the authors) after 8 h of incubation of the cells with the proteasome inhibitor. These changes have obvious consequences from the point of view of the regulation of glycolysis and can only be explained by increased protein synthesis, likely due to increased gene transcription, pre-mRNA processing or translation of the corresponding PFK mRNAs. Kim et al. [27] show an increase in HIF1α levels by immunoblot analysis, not observed by the SILAC-MS experiments because HIF1α is a low abundance protein not detected with the proteomic technologies. Accordingly, the changes in PFK protein levels could be attributed, at least in part, to an increase in gene transcription due to the stabilization of HIF1α by proteasome inhibitors, as HIF1α is a well-known activator of PFK gene transcription during hypoxia [30]. The effects reported by Udeshi et al. [28] after treatment of the cells for 4 h with 5 μM of a less specific proteasome inhibitor, MG132, are not as extensive in the whole proteome but still are highly significant. The expected effects on protein levels by treatment of cells with proteasome inhibitors are time-dependent as transcription, processing, and translation are needed before a change in protein levels can be observed, and 4 h is enough time to observe those effects translated into protein levels.

The above analysis further strength our conclusion, always consider possible effects on transcription and translation in the interpretation of the results obtained when using inhibitors of protein degradation, as exemplified with proteasome inhibitors. The rational of this consideration seems obvious for experiments with a prolonged time of incubations (steady state) but also applies for time-course experiments that require >2–4 h of treatment of the cells with those inhibitors to observe a significant effect in protein levels. A similar scenario is encountered in experiments where the effects of so-called "toxic" proteins accumulated in neurodegenerative diseases are studied. Those "toxic" proteins have clear effects on gene transcription and posttranscriptional regulation of mRNA, independent of their possible effects on protein degradation [31–33]. In summary, the contribution of changes in the copy number of mRNA molecules and translational rate has always to be taken into account for the correct interpretation of the results of experiments with protein degradation inhibitors or expression of "toxic" proteins under steady-state conditions and in time-course experiments.

The most common kinetic experiments are usually performed by treatment of cells with protein synthesis inhibitors (cycloheximide, emetine, and anisomycin) provided that cell toxicity is controlled by the analysis of cell viability along the time course of the experiment. In this experimental approach, translation is surely inhibited, but also RNA pol I and RNA pol III transcription are inhibited, without affecting general RNA pol II transcription [34], actually some pol II genes can even increase its transcriptional rate in the presence of protein synthesis inhibitors. Besides those effects, there is also a cell response to translational arrest. Protein synthesis inhibitors have also a very potent activating effect on stress kinases, JNK, and SAPK [35] and also ERK1/2 in the presence of growth factors [36] that leads to the activation of c-fos, fos B, c-jun, junB, and jun D and transcription of genes regulated by those transcription factors. Accordingly, if the protein under study is subjected to PTM by those stress kinases, both the effect of protein synthesis inhibition and changes in PTMs of the protein, likely affecting also its PPIs, could be implicated in the rate of degradation observed under those conditions. The role of PTM and PPIs in the regulation of the degradation rate of proteins has many examples in the literature [37], and their effects on a proteomic scale are beginning to be studied [22, 29]. The point made here is that PTMs and PPIs have always to be considered as a possible mechanism that may modify the interpretation of the results obtained by the use of protein synthesis inhibitors.

Another possible situation is that the protein under study is poorly degraded even after prolonged incubation of the cells with protein synthesis inhibitors. Those results could be due to the actual fact that the protein under study has a very long half-life or alternatively that a protein with a shorter half-life is required to interact with the specific protein under study in order to be targeted to degradation. It is also noteworthy to recall that protein synthesis inhibitors also affect the machinery of degradation, with inhibitory effects on the multicomponent ubiquitin-proteasome [38–40] and autophagic pathways [41]. The combined use of protein synthesis and degradation inhibitors in time-course experiments is the most usual experimental setup to study the role of different proteolytic degradation pathways, and the considerations made above should also be taking into account for the interpretation of the results of these combined pharmacological experiments.

3.2. Genetic Interference Studies of Protein Degradation. Transcriptional shut-off (Tet-on, Tet-off) by tetracycline responsive promoters of protein expression [42] and mRNA translational inhibition and/or mRNA degradation using siRNA or shRNA [43] are the main genetic methods to knock-down the mRNA levels encoding for the protein whose degradation is being investigated or for determining the role of a protein in the degradation of another protein.

The correct interpretation of the results of these genetic interference methods requires understanding that mRNA degradation, and/or translation inhibition, is a time-dependent process. Accordingly, a new steady state of the protein levels will be attained concomitantly with the decay of the corresponding mRNA and/or its translational inhibition, either after transcriptional shutdown of gene expression (Tet-on/off systems) or by inhibition of translation and/or degradation in the case of siRNA and shRNAs. Using the spreadsheet available on-line, the reader can model different experimental situations to simulate this type of experiments. If the half-life of the mRNA is much shorter than the actual half-life of the protein, the experimental results obtained in a shut-off transcriptional or RNA interference experiment will allow the accurate determination of the half-life of the protein. In any other situation, the mRNA half-life (and/or the rate of translational inhibition) will determine the rate of disappearance of the protein measured by immunoblot of total cell extracts. As a consequence, to correctly interpret the results obtained in both types of genetic interference setups, the rate of decay of the mRNA and/or translation inhibition (easily determined by pulse radioactive experiments and immunoprecipitation) has to be evaluated. Unfortunately, those control experiments are frequently not considered when using those methodologies. As an example, this criticism applies to the studies of alpha-synuclein half-life in cells using the Tet-on/off methodology [44, 45]; those studies do not take into account that their results on the half-life of the protein are affected by the rate of decay of the alpha-synuclein mRNA. As a consequence, their conclusions about the regulation of the degradation of alpha-synuclein are not correctly validated.

4. Pulse-Chase Experiments

Pulse-chase experiments are done either with the traditional radioactive aminoacids (^{35}S-Met, Cys, or ^{14}C aminoacids) followed by total radioactive count or immunoprecipitation with specific antibodies to the protein under study or the heavy/light isotope pulse labelling, SILAC, followed by mass spectrometry- (MS-) based quantitative proteomics (see comments above, under steady-state versus kinetic studies). Traditional pulse-chase experiments measure the rate of degradation of newly synthesized proteins. The newly synthesized proteins followed a maturation process (folding and in most cases oligomerization) and may be subjected to specific PTMs and could present unique PPIs that may differ considerably from those of the preexisting proteins (already mature proteins) in the cell. As a consequence, is conceivable that the rate of degradation of the newly-synthesized (labelled) proteins may be different from the rate of degradation of the proteins pre-existing in the cell as measured, for example, by treatment of the cells with protein synthesis inhibitors. There are factors that must be taken into account in those traditional pulse-chase experiments. Before the pulse with the radioactive aminoacids, cells are usually starved for aminoacids during variable time periods to deplete the endogenous pool of the aminoacid that will be used for protein labelling. During the starvation period cells, respond to aminoacid starvation by activating the ubiquitin-proteasomal pathway earlier than the activation of the autophagic pathway, producing an increase in protein degradation rates of pre-existing proteins in order to ensure the continuation of aminoacid supply for de novo protein synthesis [46]. Proteasome inhibitors are usually tested in this experimental setup to inhibit protein degradation by the ubiquitin-proteasome pathway, with the effects already described in the previous section. As a consequence, keeping to a minimum, the time of aminoacid starvation and pulse-radioactive labelling (total 30 min–2 h) is a good experimental practice. Furthermore, if proteasome inhibitors are used (as discussed above) it may be required to perform pulse-chase experiments in the absence and in the presence of protein synthesis inhibitors during the chase period, to control for the possible increase in mRNA abundance due to transcriptional and posttranscriptional effects of proteasomal inhibitors.

5. Using Tagged Proteins

In many occasions when antibodies against the protein under study are not available, or in order to study the effect of mutations on protein stability, expression of proteins with a tag is used to study protein degradation. This technique allows easy identification of the protein by immunoblot or by immunoprecipitation with specific antitag antibodies. Another variant is the use of fusion constructs of the protein of interest with fluorescent or luminescent proteins. The general assumption behind those experiments is that the fusion of the protein of interest with any tag will show the same behaviour as the untagged protein. Any protein fused to a "big" tag, like GFP or luciferase or "medium" tags, like TAP (Tandem Affinity Purification), is "suspect" of misbehaviour from the point of view of its degradation rates, unless experimental evidence shows the opposite. This situation is especially critical when the degradation rate of the whole proteome is being evaluated with the "big" and "medium" tag methodology. The above caveat has already been pointed out in a recent publication [47], where the authors compare the estimated half-lives obtained by the ratios of GFP-fused proteins [48] and by SILAC/MS-based quantitative proteomics [19], showing big discrepancies. Another caveat with the use of tagged proteins, even if there are "small tags" as Flag, HA, Myc, V5, and so forth, is the location of the tag within the protein sequence. Most often the tag is fused either to the N- or the C-terminus of the protein under study. Very few studies take into consideration that the tag may affect the protein structure, its PTMs and PPIs or even its subcellular localization and rate of degradation. We will illustrate here the relevance of N- and C-terminal tagging with our own studies on DJ-1 degradation, a dimeric protein whose mutations are associated with early onset Parkinson's disease [49]. One of those mutations L166P disrupts dimer formation and the mutant protein has no significant secondary structure being a direct substrate for the 20S/26 proteasome [49]. In the studies of the degradation of the mutant DJ-1 L166P by

cell transfection, we have compared the degradation of the untagged DJ-1 L166P with the N-terminal flag tagged and C-terminal V5-flag tag version in HeLa cells. As shown in Figure 2, the half-life of the untagged protein is rather short. Tagging the protein with Flag-tag at the N-terminus greatly increase the half-life of the protein, while tagging at its C-terminus with V5 flag produced no significant effect. Accordingly, the behaviour of N-tagged and untagged DJ-1 L166P proteins cannot be expected to be the same. In summary, if possible, for protein degradation studies (actually in any type of studies) is better to use untagged proteins and express them to levels not very different from the endogenous expression levels. When unavoidable, experiments should be performed with proteins tagged at the N- and C- terminus to clarify that the results are independent of the location of the tag.

6. Subcellular Localization and Protein Degradation

There are many examples from the literature showing that the degradation of certain proteins can take place in different cell compartments by different mechanisms, and those proteins change their subcellular localization (determined by PPIs and PTMs) after a cell stimuli or stress, and a special case, as mentioned in the introduction, is neurons [9]. The ideal experimental setup to study protein degradation will be to quantitate the rate of degradation of a protein directly within its subcellular compartment (nucleus, cytoplasm, mitochondria, etc.) by a direct method. The problem is similar to determine the actual rate of an enzyme in the cell without disrupting the cell for biochemical analysis, only NMR imaging can do it and still for a few enzymatic reactions. The use of fluorescent fusion proteins allows direct imaging and quantitation, but the caveats of this experimental approach for studying protein degradation has already being mentioned (see above), both at a proteomic scale and for studying a single protein. Nevertheless, the use of tagged proteins seems technically unavoidable at present. New semi- or noninvasive cell techniques are needed to be able to study the "in situ" degradation rates of proteins within its subcellular location in intact cells.

7. Checklist for Critical Appraisal of Protein Degradation Studies

A simple way to evaluate studies on protein degradation is to have in mind, or at hand, a simple scheme like the one presented in Figure 1 and to ask the following question. How many boxes of Figure 1 are not considered (lack of information) in the interpretation of the data obtained or presented? This is followed by the question: any of the processes in those boxes, black boxes in the study because of lack of information, are likely to affect interpretation of the results? The rule of the thumb is as follows: as many black boxes the study has, as much uncertainty will have the interpretation of the results. Nothing spectacular, everybody knows it.

Here we are providing a checklist for a critical appraisal of the design and interpretation of protein degradation studies. The main points to consider when analyzing a study of the role of protein degradation on the changes in protein cellular levels are as shown in Table 1.

Note that the experimental evidence obtained with the different methodologies, excluded the SILAC/MS steady-state experiments, is only partial and requires further experimental data and controls. Specific comments of the checking list for the analysis of an experiment or project on protein degradation follows.

Tick on 1. Studying the degradation of untagged proteins is the best approach to begin with the study of the degradation of a protein. Preferentially the endogenous levels of the protein in the cell should be studied. Alternatively, DNA constructs of the protein can be transfected in cells with a gene knock out for the protein under study. In this latter case, the expression levels of the transfected protein construct should be similar to those present in wild-type cells. The latter is also a good approach to study stability of mutant proteins in the absence of expression of the wild-type protein. The transfection experiments in knock-out cells are of little use if the lack of expression of the protein under study is known, or expected, to alter significantly the proteome or cell proteostasis by any mechanism, unless those are the objectives of the research.

Tick on 2. Using tagged proteins is not a very good approach. If unavoidable, perform controls with tagging at the N- and C-terminus of the protein and check if the results differ significantly.

Ticks on 1 or 2, 3 or 4, and 7. Tagged or untagged protein steady-state or kinetic experiments with inhibitors of protein degradation. *The changes in the protein levels cannot be attributed to changes in protein degradation rate*. Compulsory to do control experiments: to measure the amounts of mRNA, especially if proteasome inhibitors are used (they affect transcriptional rates), for the protein of interest and even the rate of translation of its mRNA. Treatment with inhibitors of protein degradation may also affect PTM of the protein under study (PPIs and subcellular localization may be also affected); remember protein kinases and phospatases are also subjected to control by degradation.

Ticks on 1 or 2, 4, and 5. Tagged or untagged protein kinetic experiment with inhibitors of protein synthesis. *The changes in protein levels may be attributed to protein degradation of pre-existing protein in the cell.* Additional required evidence should be provided as follows: cell viability during the time-course experiments and also that there are no changes of PTMs, PPIs, and subcellular localization of the protein under study in the presence of protein synthesis inhibitors.

Ticks on 1 or 2, 4, 5, and 7. Tagged or untagged protein kinetic experiment with inhibitors of protein synthesis and degradation. *The changes in protein levels may be attributed to protein degradation of pre-existing proteins in the cell.* The inhibitors of protein degradation can be added at the same time as protein synthesis inhibitors or earlier. Prolonged preincubation periods of the cells with inhibitors of degradation should be

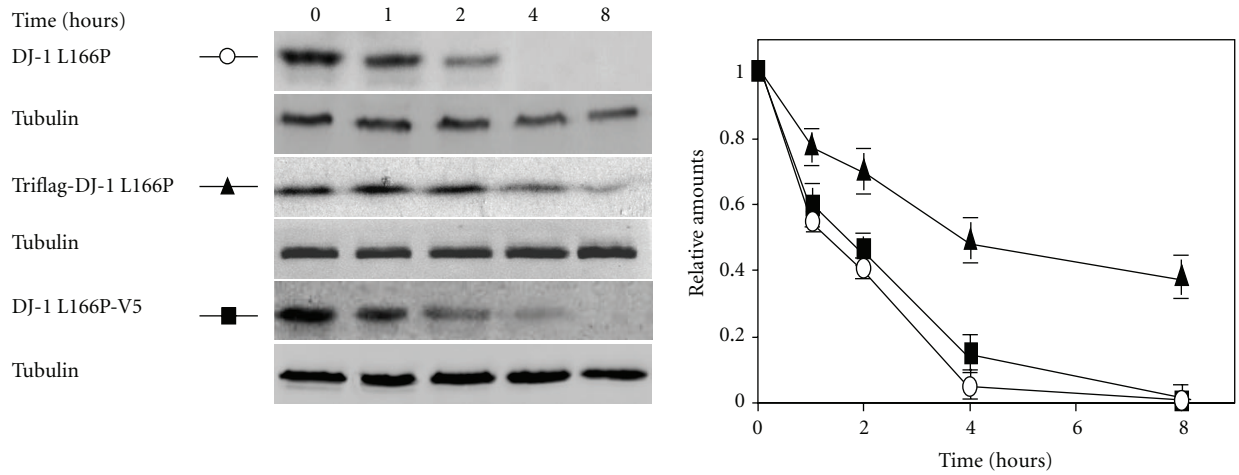

Figure 2: Degradation of untagged and N-terminus and C-terminus tagged versions of human DJ-1 L166P. The untagged human DJ-1 L166P (hDJ-1 L166P) construct has been described previously [49]. The C-terminal His-V5 tagged hDJ-1 L166P was obtained by PCR amplification with the following oligonucleotides (forward BamH1-DJ-1; 5′GGAAGGATCCATGGCTTCCAAAAGAGCTCTGG 3′ and reverse Nonstop-hDJ-1; 5′GTCTTTAAGAACAAGTGGCGCCTTCACTTGAGC 3′) and cloned into pcDNA 3.1/V5-His Topo vector from Invitrogen The N-terminal 3xFlag- tagged hDJ-1 L166P was obtained by PCR amplification with the following oligonucleotides (forward BamH1-hDJ-1 and reverse XhoI-hDJ-1 5′GCGCCTCGAGCTAGTCTTTAAGAACAAGTGGAGCC 3′) and cloned into the pCMV-3Tag 1-A vector. N2a cells were cultured in DMEM medium with 10% FBS and transiently transfected with the different hDJ-1 L166P constructs. Transfected cells were treated with cycloheximide (20 μg/mL) for the times indicated and analyzed by Western immunoblotting with anti-hDJ-1specific antibodies, as described in [49]. Results presented are expressed as mean ± s.e.m for at least three independent experiments.

Table 1

		Type of study	Conclusion
1	☐	Untagged protein	Good approach
2	☐	Tagged protein	Not a good approach
3	☐	Usual steady state	Partial evidence
4	☐	Kinetic (time-course)	Partial evidence
5	☐	Protein synthesis inhibitor	Partial evidence for pre-existing proteins in the cell
6	☐	Radioactive pulse-chase experiment	Partial evidence for newly synthesized proteins
7	☐	Inhibitors of protein degradation	Partial evidence
8	☐	Tet-on Tet-off, RNA interference	Partial evidence
9	☐	SILAC-MS steady-state experiments under basal conditions (turn-over)	Good evidence
10	☐	SILAC-MS upon cell stimulus or stress	Partial evidence

avoided, as it may change the levels of the protein due to changes in transcription, mRNA stability, translation, and so forth. Furthermore, long preincubation may also alter the expression levels of many proteins, possibly resulting in a modification of PTMs or PPIs of the protein under study, obviously affecting its rate of degradation. Always consider that PTMs, PPIs, and subcellular localization can be affected by the use of protein synthesis and degradation inhibitors.

Ticks on 1 or 2, 4, and 8. Tagged or untagged protein kinetic experiment with genetic interference. *The decrease in protein levels cannot be attributed exclusively to protein degradation.* Compulsory to measure the decay (time-course) of the mRNA (Tet-on/Tet-off experiments), and decay of mRNA and/or translational inhibition (RNA interference experiments), in order to be able to interpret the results. Perform other kinetic experiments to validate the data obtained. The on-line spreadsheet can be used as a help to interpret the results.

Ticks 1 or 2, 4, 7, and 8. Tagged or untagged protein kinetic experiment with genetic interference and inhibitors of protein degradation. *The changes in protein levels cannot be attributed exclusively to protein degradation.* Compulsory to measure rate (time-course) decay of the mRNA (Tet-on/Tet-off experiments) and decay of mRNA and/or translational rate (RNA interference experiments) in the absence and in the presence of protein degradation inhibitors. The on-line spreadsheet can be used as a help to interpret the results.

Ticks on 1 or 2 and 6. Tagged or untagged protein using pulse-chase radioactive experiments. *The decrease in the levels of*

the labelled protein can be attributed to degradation of the newly synthesised protein. The rate of degradation of the newly synthesized protein may not be identical to the rate of the degradation of the pre-existing protein in the cell. The rates will be similar (or identical) when protein folding, PTMs, PPIs, and subcellular localization of the newly synthesized protein are much faster processes than the rate of degradation of the protein.

Ticks on 1 or 2, 6, and 7. Tagged or untagged protein using pulse-chase radioactive experiments with inhibitors of protein degradation. *The changes in the levels of the labelled protein can be attributed to degradation of the newly synthesised protein.* The rate of degradation of the newly synthesized protein may not be identical to the rate of the degradation of the pre-existing protein. The inhibitors of protein degradation are routinely added at the beginning of the chase period, and they can produce a change in the amount of mRNAs or mRNA translational rate (see above) producing a faster chase and decreasing the estimated value of the half-life of the protein. Addition of protein degradation inhibitors during the aminoacid starvation before the pulse or along with the pulse may be needed for proteins with short half-life (<2 h). In those cases, again, the presence of protein degradation inhibitors could affect the rate of protein synthesis and as a consequence, the initial amounts of the radioactive protein under study could be different respect to the controls. Under these experimental conditions is generally assumed that inhibitors of protein degradation do not affect protein folding, PPIs, PTM, and subcellular localization of the newly synthesized protein, but it could not be the case.

Ticks on 1 or 2, 5, 6, and 7. Tagged or untagged protein using pulse-chase radioactive experiments with inhibitors of protein synthesis and degradation. *The changes in the levels of the labelled protein can be attributed to degradation of the newly synthesised protein and can be compared with the degradation rate of pre-existing protein in the cell.* This experimental setup allows the comparison of the rate of degradation of the newly synthesized protein (pulse-chase) and that of the pre-existing protein by addition of the protein synthesis inhibitors during the chase period. In essence, performing immunoprecipitation experiments of radioactive total cell extracts together with determination of total protein levels by immunoblot analysis. If the degradation of the newly synthesized protein is inhibited by the presence of protein synthesis inhibitors, it could indicate that another protein(s) with a shorter half-life than the protein under study is required for targeting the protein under study to degradation. Alternatively, it could indicate that PTMs or PPIs changes in response to protein synthesis inhibitors affect the degradation of the newly synthesized protein. Apply also the same cautions respect to the use of inhibitors of protein degradation as in previous entries.

Ticks on 1 or 2 and 9. Tagged or untagged protein using steady-state SILAC/MS experiments. *The calculated changes in the levels of the labelled protein can be attributed to protein degradation under steady-state conditions.* By comparison of the rates of disappearance of the heavy/light and appearance of the light/heavy peptides after the shift in the SILAC experiments, the degradation rate of a protein under steady-state conditions can be estimated. This methodology seems the best suited to estimate protein turnover under unperturbed cell conditions. The half-life values obtained are average values resulting from the interplay of the rates of the different processes involved in protein homeostasis (Figure 1). At present, the results reported by different groups have strong variability that can be due to the use of different cell lines, but it might also reflect technical problems related to handling, processing of the samples, MS sequence coverage of the proteins, and also to calculations.

Ticks on 1 or 2 and 10. Tagged or untagged protein using kinetic SILAC/MS after cell stimulus or stress. *The calculated changes in the levels of the labelled protein cannot be attributed to protein degradation exclusively.* Any perturbation of the steady state of a cell by a stimulus or stress, like treatment of cells with inhibitors of protein synthesis or degradation, needs to show that the changes in degradation rates cannot be explained by changes in other proteostatic processes (Figure 1), changes in the protein abundance due to transcriptional, posttranscriptional processing of RNA (including mRNA transport and decay), or changes in protein synthesis, folding, oligomerization, PTMs, and PPIs as a consequence of the stimuli or stresses applied to the cells.

8. Conclusion

Interpretation of studies of protein degradation requires, as with other biological experiments, a critical assessment of the methodology and the data obtained. A rigorous analysis will prevent misleading conclusions. Examining the data obtained by asking a series of simple proteostatic questions can uncover serious deficiencies. Sometimes it could be difficult to interpret the data, and to present a balanced and impartial summary may be not an easy task. However, failing to do a critical analysis is damaging for science. This situation is especially relevant in a time of massive "omic" data availability. The incorrect interpretation of the enormous amounts of data obtained, together with the postdata computational analysis by merging of actual data on protein degradation with human classifications of the properties of proteins (GO terms, pI, unstructured elements, molecular weight, etc.), may produce many papers and holistic conclusions that in a few years could generate a completely new whole field of science "mislead omics."

Note 1. Since this review was submitted, a report has appeared [50] describing that DJ-1 L166P promotes cell death by dissociating Bax from Bcl-XL. Unfortunately the authors used N-Terminal Flag DJ1 L166P for their experiments, and as shown here Flag-DJ-1 L166P (Figure 2) has a reduced rate of degradation compared to the untagged version, and as a consequence has higher cellular steady-state protein levels than the untagged DJ-1 L166P. As PPIs are governed by Law of Mass Action (equilibrium constant), the interactions

reported for the N- Flag-DJ-1 L166P may not be quantitatively relevant for the natural (untagged) DJ-1 L166P. Accordingly, the effect of DJ-1L166P on apoptosis would be minimal or even not existent.

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Review Article

The Ubiquitin-Proteasome System in Huntington's Disease: Are Proteasomes Impaired, Initiators of Disease, or Coming to the Rescue?

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Huntington's disease is a progressive neurodegenerative disease, caused by a polyglutamine expansion in the huntingtin protein. A prominent hallmark of the disease is the presence of intracellular aggregates initiated by N-terminal huntingtin fragments containing the polyglutamine repeat, which recruit components of the ubiquitin-proteasome system. While it is commonly thought that proteasomes are irreversibly sequestered into these aggregates leading to impairment of the ubiquitin-proteasome system, the data on proteasomal impairment in Huntington's disease is contradictory. In addition, it has been suggested that proteasomes are unable to actually cleave polyglutamine sequences *in vitro*, thereby releasing aggregation-prone polyglutamine peptides in cells. Here, we discuss how the proteasome is involved in the various stages of polyglutamine aggregation in Huntington's disease, and how alterations in activity may improve clearance of mutant huntingtin fragments.

1. Introduction

1.1. Huntington's Disease. Huntington's disease (HD) is one of nine polyglutamine (polyQ) disorders know to date, which are caused by an expansion in the CAG repeat sequence of the encoding DNA that is subsequently translated into a polyQ expansion within the disease-related protein [1, 2]. The presence of a glutamine repeat within proteins is a common feature mainly in transcription factors and may mediate in protein-protein interactions [3, 4]. However, when the polyQ repeat exceeds a length of around 37 glutamines the expansion becomes disease causing [5, 6]. The severity of the disease is correlated with the length of the polyQ expansion, as an increasing repeat length correlates with earlier onset of disease and more severe symptoms [1]. There is strong evidence that the polyQ expansion induces a gain of function since insertion of an expanded CAG sequence in the hypoxanthine phosphoribosyltransferase (HPRT) gene, an HD-unrelated gene which is not involved in any polyQ disorders, induced late-onset neurodegeneration and premature death in a mouse model similar to transgenic HD mouse models [7]. In addition, overexpression of polyQ peptides in transgenic mice caused a neurodegenerative phenotype demonstrating that the polyQ stretch by itself induces toxicity [8]. Still, a loss of wildtype huntingtin (htt) function may also contribute to the disease when considering the broad spectrum of functions which are ascribed to wildtype htt [9]. The htt protein, affected in HD, is an ubiquitously expressed protein which is proposed to be important in embryonal development, transcriptional regulation, axonal, and vesicle transport and has an antiapoptotic function [10]. Although htt is ubiquitously expressed, the earliest neuropathological changes in HD are found in the striatum and cerebral cortex, which are involved in motor control, cognition, and sensory pathways [11]. This leads to a cognitive decline in a progressive manner and manifests in motor dysfunction and severe dementia [12]. Furthermore, HD is characterized by psychiatric and emotional disturbances [13]. The fact that particular brain regions are more affected than others suggests that specific neurons are

more vulnerable to htt-induced toxicity probably due to cell-specific gene expression, protein-protein interaction, or posttranslational protein modification [14, 15].

While the exact disease mechanisms behind HD remain elusive, many cellular pathways including transcriptional dysregulation, activation of apoptotic pathways, altered neurotransmitter release, mitochondrial dysfunction, and oxidative stress were found to be affected and therefore subjected to research for therapeutic intervention [16]. An important pathological hallmark of all polyQ disorders is the presence of intracellular protein aggregates, similar as observed in other neurodegenerative disorders like Parkinson's disease and Alzheimer's disease. In the case of HD, aggregates found in human HD postmortem brain are composed of mutant htt (mhtt) N-terminal fragments containing the polyQ stretch [17, 18]. The N-terminal mhtt fragments are highly prone to aggregate in the cell, and accumulating evidence suggests that especially small aggregates of oligomeric mhtt cause cellular toxicity [19–21]. Improving the clearance of these intermediate aggregates or monomeric mhtt fragments should therefore be a therapeutic target to prevent or delay the onset of HD.

1.2. The Ubiquitin-Proteasome System. The two main pathways involved in the degradation of intracellular proteins are the ubiquitin-proteasome system (UPS) and autophagy. Degradation via the UPS is essential for the clearance of short-lived and misfolded proteins, while autophagy mostly targets long-lived proteins and large structures like protein aggregates or organelles [22, 23]. Both cellular pathways are involved in polyQ protein clearance but at different levels. Degradation of mhtt via macro-autophagy requires targeting of proteins towards lysosomes, which is initiated by engulfment of proteins into autophagosomes. These subsequently fuse with lysosomes to form autolysosomes, resulting in breakdown of their contents by hydrolytic enzymes [24, 25]. However, aggregates of N-terminal mhtt fragments are mainly present in the cell nucleus in human HD postmortem brains [17, 18], while macro-autophagy is a cytoplasmic degradation pathway and therefore not sufficiently effective in clearing nuclear mhtt aggregates. To target nuclear mhtt fragments, the UPS gets into the picture, as proteasomes are present in both the cytoplasm and nucleus. Indeed, various studies indicate that the UPS is involved in processing both wildtype and mhtt [26, 27]. The UPS is mainly involved in maintaining cellular homeostasis via degradation of short-lived regulatory proteins like transcription factors and cell cycle regulatory proteins but also has a protective function since it is responsible for the degradation of damaged and misfolded proteins [28]. Most proteins designated for destruction by the UPS are first tagged by a polyubiquitin chain, which is an ATP-dependent process that occurs via a three steps process. First, Ubiquitin (Ub) is activated by an E1 ubiquitin-activating enzyme, followed by binding to an E2 conjugating enzyme, and finally the binding of the Ub moiety to a lysine residue within the targeted protein via an E3-ligase. Subsequent ubiquitination of the conjugated Ub leads to a poly-Ub chain which designates the protein for targeting towards the 26S proteasome, where the substrates are recognized, unfolded, and degraded [29]. The 26S proteasome includes two major complexes, the 20S core proteasome and the 19S regulatory particle. The 19S regulatory particle recognizes and de-ubiquitinates the polyubiquitinated substrate, unfolds the protein, and guides it through the 20S core [30–32]. The 20S core is a cylindrical complex consisting of four rings stacked on top of each other, while each ring contains seven subunits [33–35] (Figure 1(a)). The two outer rings consist of α-subunits that close the interior of the barrel shaped complex, whereas the inner two rings are composed of seven β-subunits including three subunits with catalytic activity. These three active subunits, referred to as $\beta1$, $\beta2$, and $\beta5$, have caspase-like activity which cleaves behind acidic residues, trypsin-like activity which cleaves after basic residues, and chymotrypsin-like activity which cleaves behind hydrophobic residues, respectively. When unfolded substrates enter the hollow cavity of the 20S complex, their amino acid chains are then attacked by the N-terminal threonine residue of the catalytic subunits [33, 34, 36, 37] (Figure 1(b)). After cleavage, peptides are released into the cellular environment, where they are further processed by peptidases for antigen presentation or recycled into amino acids.

2. The Role of the Proteasome in Huntington's Disease

2.1. Proteasomes in HD: The Good, the Bad, or the Ugly? Various studies indicate that the UPS is involved in processing mhtt since aggregates induced by mhtt are positively stained for Ub and proteasome subunits in human HD postmortem brains, in HD transgenic R6/2 mice that express polyQ-expanded mhtt-exon1(Q145) and in cell culture [17, 38–40]. Also soluble mhtt is polyubiquitinated in cells transfected with mhtt and in HD patient material, suggesting that mhtt can be targeted by the UPS [41–43]. Though, recently it was shown in cell culture that mhtt inclusions are initially devoid of ubiquitin and that soluble mHtt is not extensively ubiquitinated [44]. Furthermore, *in vitro* data suggested that proteasomes may actually be unable to degrade the polyQ repeat present in proteins, as purified mammalian 26S proteasomes were only able to cleave within the flanking sequences or after the first glutamine of a polyQ-containing peptide, while the remaining polyQ repeat was released by the proteasome [45]. One possible consequence of the ineffective degradation of polyQ sequences could be the clogging of proteasomes by long polyQ repeats. Proteasomes generate peptides with an average length of 3–9 amino acids, and these peptides do not exceed a length of 22 amino acids [46]. When confronted with a polyQ-expanded protein, the undigested polyQ peptide is much longer, which may then be unable to diffuse out of the narrow α-pore thereby clogging the proteasome, resulting in proteasomal impairment. This hypothesis was supported by FRET experiments showing a stable interaction between the proteasomal catalytic immunosubunit LMP2 and mhtt, although it should be noted that the proteasomal

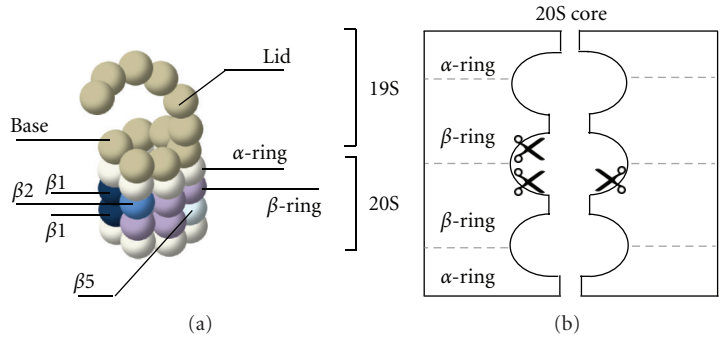

Figure 1: Representation of the 26S proteasome. (a) The 26S proteasome consists of a 20S core capped by one or two 19S activators. The catalytic subunits $\beta 1$, $\beta 2$ and $\beta 5$ are represented in blue in the β-ring. (b) A schematic model of the 20S core, indicating the presence of cleavage sites inside the barrel-shaped structure.

fluorophore was on the outside of the proteasome core, and may thus reflect proteasome binding to htt aggregates [47]. An alternative consequence would be the release of the polyQ peptides generated by the proteasome into the cellular environment, which subsequently would initiate aggregation. Indeed, when mimicking this polyQ peptide release in cells, polyQ peptides exceeding the disease-related threshold of around 40 glutamines showed resistance to degradation, leading to their accumulation and subsequent aggregation [48]. To prevent their accumulation, proteases and peptidases downstream the proteasome should target these polyQ peptides. One of the peptidases shown to be able to target polyQ sequences is puromycin-sensitive aminopeptidase (PSA), which is however, only able to degrade peptides up to 33 amino acids in length [49]. Surprisingly, PSA could still reduce aggregation and toxicity induced by polyQ-expanded peptides or mhtt, but this appears to occur via activation of the autophagy pathway and not via direct degradation by the peptidase [50]. Together this data indicates that proteasomes cannot process polyQ fragments, which consequently could result in proteasome impairment.

Indeed, various studies in cell models and in patient material have reported that the UPS is impaired in HD, which could be the underlying cause of the neurotoxicity. To examine the UPS pathway at different stages of mhtt degradation, a combination of different assays was used to detect alterations in the UPS in striatal cells derived from HttQ111 knock-in mice which express full-length mhtt at endogenous levels [51]. By using small fluorogenic proteasome substrates, as well as various short-lived luciferase reporters which act at different levels of the UPS, it was shown that the UPS is affected at two different levels. A change in activity of the 20S proteasome was detected, as the caspase-like and chymotrypsin-like activities were downregulated, whereas the trypsin-like activity was highly upregulated. Importantly, no effect on the degradation of short-lived proteins that did not require ubiquitination was detected, whereas an increase in the half-life of a polyubiquitinated reporter was observed, indicating that there was a defect in recognition, deubiquitination, or unfolding by the 19S cap. Since an increase in trypsin-like activity was also observed after a stress response upon ATP depletion, it was suggested that expression of mhtt may have caused this change in proteasome activity by a similar, indirect mechanism. In agreement with these results, a decrease in proteasomal caspase-like and chymotrypsin-like activity was also detected both in postmortem brain material and in skin fibroblasts of HD patients, by using small fluorogenic peptides [52]. Together, these studies suggest an overall proteasome impairment both in cells and patient material.

Furthermore, coexpression of mhtt with proteasomal subunits in cells also revealed that recruitment of proteasomes into HD aggregates seems to be irreversible, as fluorescence recovery after photobleaching (FRAP) experiments in living cells using fluorescently-tagged proteasomes showed no recovery of bleached proteasomes that resided in aggregates [47]. These findings led to the conclusion that proteasomes are trapped into polyQ aggregates, which would lead to impairment of the UPS due to Ub and proteasome depletion and even direct blockage or clogging of proteasomes. When using small fluorogenic substrates to quantify proteasome activity, a decrease in proteasomal activity was detected in the soluble fraction of neuronal cells stably expressing an N-terminal fragment of mhtt-Q150, whereas an increased proteasome activity was detected in the insoluble cell fraction containing aggregates [53]. Since there was also a decrease in the degradation of the proteasomal substrate p53, it was concluded that the sequestration of proteasomes into aggregates caused impaired proteasome functionality and neurotoxicity in the cell. This impairment due to sequestration in aggregates was further confirmed by groups using a short-lived GFP^u reporter, which has a CL-1 degron signal fused to the C-terminus of GFP, thereby targeting GFP^u for proteasomal degradation [54]. When this UPS reporters was cotransfected with polyQ proteins in HEK293 cells, intracellular GFP fluorescence increased 2-3-fold compared to control cells, indicating that proteasome impairment occurred in polyQ protein-expressing cells [55]. The increase in fluorescence and thus proteasome impairment was even higher in polyQ aggregate-containing cells, although it cannot be excluded that this could be due to higher expression levels of the introduced cDNAs in these cells. A global proteasome impairment was

reported when mhtt aggregates were present in either the nucleus or the cytoplasm, using GFP^u reporters fused to NES or NLS signals to study proteasomal activity in only the cytoplasm or the nucleus of HEK293 cells [56]. While aggregates were present in *trans* compartments, this still led to an increase in GFP fluorescence, suggesting that the UPS was globally affected. Interestingly, this *trans* impairment did not require the presence of mhtt aggregates but also occurred at an earlier stage, indicating that sequestration in aggregates is not a requirement for UPS impairment. Furthermore, this study also showed *in vitro* results which contradict proteasome clogging by mhtt, since purified mhtt aggregates completely failed to impair proteasomes.

Despite the experiments with purified proteasomes, showing the inability to cleave within polyQ sequences which could lead to proteasomes clogging or continues engagement while trying to degrade mhtt, there are various reports suggesting that proteasomes are capable to digest polyQ sequences. First, proteasomal inhibition increases mhtt levels and in some cases even to a larger extent than macroautophagy inhibitors, although this could also be due to the accumulation of other polyubiquitinated proteins that would co-aggregate and accelerate intracellular aggregation [27]. Secondly, when using degradation signals to target polyQ proteins towards the proteasome less aggregation was observed, indicating that the proteasome can handle polyQ proteins. For example, when an ornithine decarboxylase (ODC) sequence was used to destabilize mhttQ73 in HEK293 cells, an Ub-independent degradation of mhtt was observed, suggesting that the proteolytic activity of the 20S proteasome was not the limiting factor in mhtt degradation [57, 58]. Similarly, when applying the N-end rule to test whether the UPS is capable of unfolding and degrading Ub-R-polyQ-GFP, a complete and efficient degradation of the polyQ protein, without impairment of the proteasome, was shown [59]. Thirdly, when using a NLS signal to target mhtt to the nucleus (thereby excluding clearance by autophagy), proteasomal degradation of mhtt was facilitated by the nuclear E3-ligase UHRF-2 in stable HeLa cells [42]. This E3-ligase seems to be responsible for ubiquitination of nuclear mhtt and can reduce mhtt aggregation via proteasomal degradation of soluble htt.

As it appears, proteasome impairment in mhtt-expressing cells remains controversial, and the above mentioned studies showing proteasome impairment have been challenged as well. Using short-lived polyQ-containing proteins that are rapidly targeted for proteasomal degradation via the N-end rule pathway, it was shown that these polyQ proteins were efficiently degraded when targeted towards the proteasome unless these proteins were aggregated [60–62]. Additionally, proteasome activity reporters carrying the N-terminal degron signal but not the polyQ repeat were efficiently degraded in polyQ aggregate-containing cells, implying that proteasomes were still functional in these cells but could not degrade aggregated proteins [60]. Moreover, when examining proteasome activity levels in brains of the conditional HD94 mouse model, which expresses an inducible chimeric mouse/human $httQ94^{(exon1)}$ in the forebrain, the earlier reported UPS impairment could not be detected [63]. But an increase in both the trypsin- and chymotrypsin-like activity was observed, similar to the increase in activities observed in cells expressing so-called immunoproteasomes (induced upon treatment with IFNγ, as discussed below). Indeed, labeling for immunoproteasome subunits confirmed the presence of immunoproteasomes in brains of HD mice. The absence of proteasome impairment was also more recently underscored in R6/2 mice crossed with transgenic mice expressing different short-lived GFP reporters. Both GFP^u and Ub^{G76V}-GFP, where the GFP protein is fused to a non-cleavable Ub acting as an Ub-fusion degradation (UFD) signal, have been used as a proteasomal activity marker [61, 64, 65]. In both mouse models, no inhibitory effect by mhtt on proteasomes was detected, contradicting the evidence for proteasome impairment in HD. How to explain these apparently opposite findings in proteasome activity?

2.2. Aggregate Formation Rescues Proteasome Function. As mentioned above, *in vitro* experiments do not show any impairment when proteasomes were incubated with isolated mhtt aggregates and although proteasomes are associated with aggregates, cells still contain a large fraction of proteasomes that are not associated [56, 66]. Together with the observation that proteasomal impairment can already occur before aggregate formation, this argues against a sequestration model. Moreover, a potential protective role was suggested for aggregates when cultured striatal neurons expressing GFP-tagged mhtt-exon1 were visualized by means of an automated fluorescence microscope and followed in time [21]. Surprisingly, neurons that formed large aggregates (called inclusion bodies or IBs) showed a reduction in diffuse mhtt in time and a prolonged survival compared to cells with a diffuse mhtt distribution but no aggregates. When the short-lived UPS reporter, $mRFP^u$, was coexpressed to determine proteasome activity in these cells, an improved survival of neurons with IBs was again observed which coincided with less proteasomal impairment [67]. Intriguingly, IB-containing cells showed a significant drop in proteasome activity just before IBs were formed. Together, this suggests that IB formation might be a protective mechanism to sequester toxic mhtt species in the cell that would otherwise impair the UPS.

Indeed, isolated aggregates do not impair proteasomes *in vitro*, unlike isolated mhtt filaments which induce a reduction in 26S proteasome activity [66]. This suggests that diffuse, oligomeric mhtt can cause proteasomal impairment, whereas IBs do not interfere with the UPS. However, Hipp *et al.* showed that proteasomes do not become clogged *in vitro* by mhtt. This study also excludes in vitro competition between ubiquitinated mhtt and other ubiquitinated proteins for 26S proteasome-dependent degradation [44]. Together, this suggests that mhtt does not directly affect proteasomal activity, but rather maintaining mhtt's solubility will place a burden on the total protein homeostasis machinery. The chaperone network would then become overloaded by aggregation prone mhtt, leading to an overload of proteins that depends on folding and a collapse of the proteolysis

network. The observed UPS impairment may therefore reflect the inability of cells to maintain protein homeostasis. This model would be in line with the observed transient accumulation of proteasome reporters in inducible HD94 mice that were crossed with transgenic Ub^{-G76V-}GFP mice [68]. Upon expression of the HD94 gene in two-month-old mature mice, a modest increase of the GFP reporter was measured in the first four weeks, indicating a decrease in UPS activity, followed by a decrease in GFP levels when aggregates appeared. When these mice received riluzole (an aggregation preventing compound) an increase in proteasomal reporter levels was again detected. These studies suggest that IB formation is not the bottle neck in progressive neurodegeneration, but that high levels of aggregation-prone mhtt can frustrate the UPS indirectly.

Still, one would expect that the observed sequestration of proteasomes into IBs would affect UPS function. Recent data from our lab suggests that proteasomes are not irreversibly sequestered in mhtt aggregates, but can still move outwards. When fluorescently-tagged proteasomes were co-expressed with fluorescently-tagged polyQ peptides in HeLa cells (green and red, resp., Figure 2), proteasomes were recruited into the polyQ aggregate. While the proteasome is present in the core of the aggregate in newly formed polyQ aggregates (Figure 2, upper panel), the proteasomes only occupy the outskirts of larger aggregates in contrast to the polyQ peptides (Figure 2, lower two panels). This suggests that polyQ fragments but not proteasomes are irreversibly sequestered. This shift to the outside of the larger aggregate probably occurs slowly in time and would explain why no rapid exchange of proteasomes could be observed by photobleaching experiments [47]. Since proteasomes are apparently dynamically recruited to mhtt aggregates, is it then possible to stimulate proteasome activity to improve its capacity to reduce the burden of mhtt?

3. Improving Activity of Proteasomes towards PolyQ Proteins

3.1. Changing Proteasomal Activity. While purified proteasomes are unable to degrade polyQ repeats, it appears that the UPS in living cells is somehow capable to degrade these polyQ proteins once they are targeted towards the proteasome. This could be due to various modulations in the UPS that occur after specific triggers from the cellular environment, such as alterations in proteasome composition or the recruitment of proteasome activators. If possible, it would be interesting to modify the proteasomal activity to increase cleavage of polyQ sequences? While the constitutive 26S proteasome is comprised of a 20S catalytic core and a 19S activator as described above, the 19S cap can be replaced by the proteasome activator (PA) 28γ or the PA28$\alpha\beta$ activating cap. Furthermore, the 20S catalytic subunits β1, β2, and β5 can be replaced by the immunosubunits LMP2 (PSMB9), LMP7 (PSMB8), and Mecl-1 (PSMB10).

The 20S core has two mechanisms to prevent random cleavage of substrates. First, there is a narrow channel, the α-annulus, which closes the catalytic proteasome core to folded proteins (Fig 1B) [69]. Secondly, the N-termini of the α-subunits form a closed gate which cannot even be entered by small substrates. Thus, for substrates to enter the 20S proteasome, opening of the α-gate is necessary. This can be achieved by the 19S cap, which recognizes ubiquitinated proteins, but also by other proteasome activators, jet via a different mechanism. PA28 α, β, and γ are homologous and thus activate the proteasome in a similar fashion. PA28α and PA28β together form a heteroheptameric ring while PA28γ forms a homoheptameric ring [70–72]. These activator rings can dock on the α-subunits of the 20S via binding of the PA28 C-termini into the pockets between the α-subunits, followed by opening of the proteasome [73, 74]. Unlike the 19S cap, the PA28 caps are ATP-independent and are unable to recognize ubiquitinated and folded proteins, but can stimulate the peptidase activity of the proteasome up to 200-fold dependent on the substrate [74–76]. PA28$\alpha\beta$ expression is induced upon IFNγ stimulation or viral infections, like multiple other genes involved in the immune response, and is therefore proposed to have an important role in antigen processing and presentation [77, 78]. When PA28$\alpha\beta$ binds the proteasome, all three catalytic activities of the proteasome are increased [79], which is not due to a direct effect on the catalytic subunits, but rather by structural change of the 20S core increasing the accessibility of the catalytic subunits [78, 80]. Furthermore, binding of the PA28$\alpha\beta$ ring will open the α-gate, increasing the uptake but also the release of peptides, which may explain the increase in generated peptides that are more suitable for MHC class I binding [81]. The function of PA28γ in the cell is more diverse, as multiple interaction partners and degradation targets have been identified confirming a role in various cellular processes including cell cycle regulation and apoptosis, both in a 20S-dependent or- independent manner [82, 83]. Proteasome activation by PA28γ mainly increases trypsin-like activity, suggesting a conformational change in the 20S core that covers the chymotrypsin-like and caspase-like site and exposes the trypsin-like site [79, 84, 85]. Despite the peptidase activity 20S-PA28γ proteasomes are able to cleave intact proteins via unstructured or linker regions [84, 86].

3.2. The Proteasome Activator PA28αβ. Expression of the proteasome activator PA28$\alpha\beta$ in patient material increased UPS function in control cells but not in HD fibroblasts, suggesting that introduction of PA28$\alpha\beta$ would not improve polyQ degradation in mhtt expressing cells [52]. However, these experiments were performed in cells already expressing mhtt, and it would be interesting to induce PA28$\alpha\beta$ at an earlier stage prior to disease onset in order to study the direct effect of PA28$\alpha\beta$ on polyQ degradation. Interestingly, PA28$\alpha\beta$ activation of purified 20S proteasomes increased degradation of short Q-peptides consisting of 10 glutamines, and degradation of short peptides with a glutamine at position P1 was increased with PA28$\alpha\beta$ present [45, 87]. The expression of PA28$\alpha\beta$ could improve polyQ degradation via two potential pathways, either by so-called hybrid proteasomes or via a two-step, sequential cleavage pathway

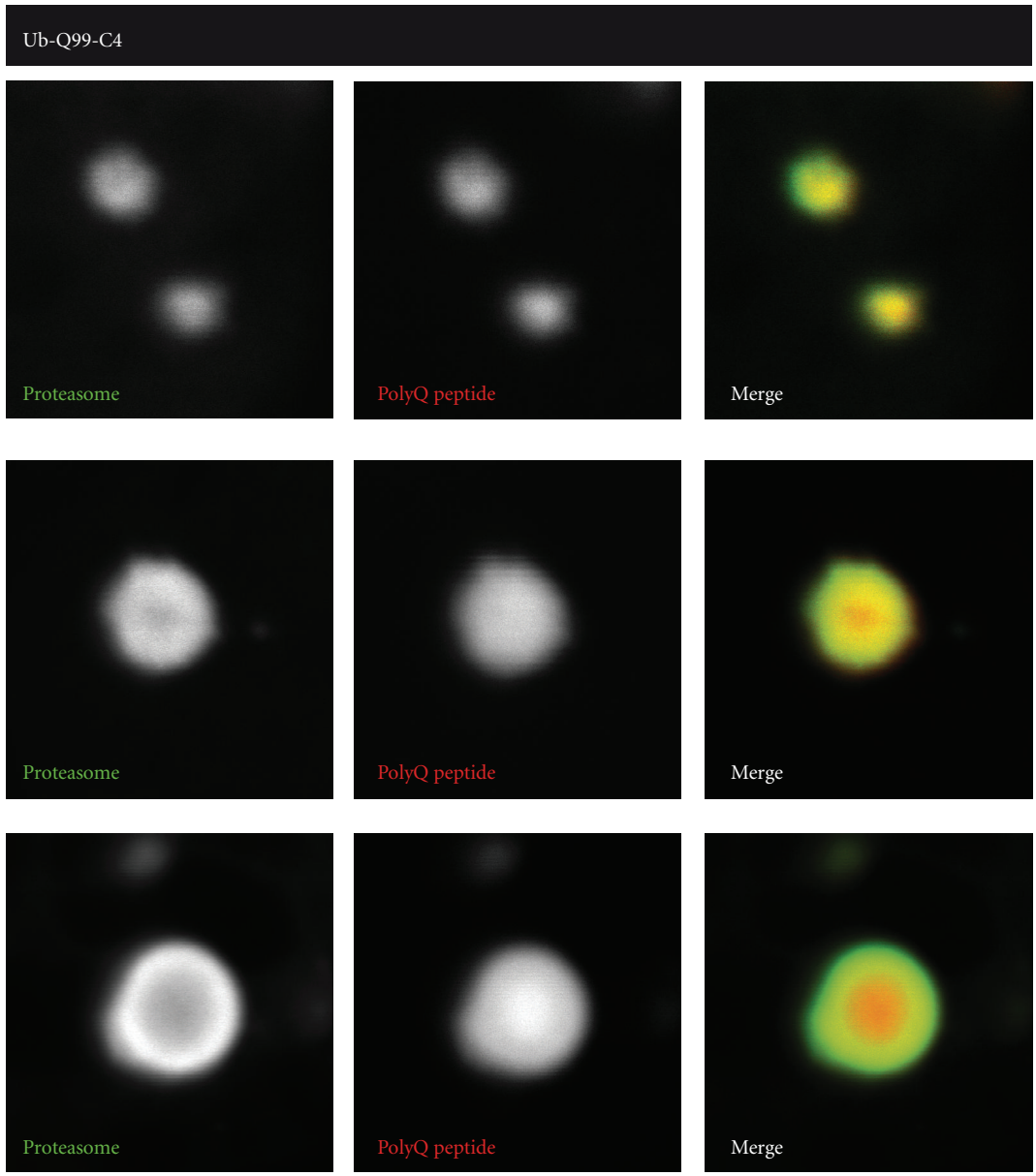

Figure 2: Localization of proteasomes in polyQ aggregates. Upon expression of red-labeled polyQ peptides in Hela cells, initially small aggregates appear (upper panel) which also recruit green-labeled proteasomes. However, in larger polyQ peptide containing aggregates the proteasome is only present in the outer layers, suggesting that proteasomes are not irreversibly trapped in aggregates.

(Figure 3). Hybrid proteasomes are composed of a 20S particle capped on one side by the 19S complex and on the other side by PA28$\alpha\beta$ [88]. Here, the 19S cap would recognize and unfold mhtt fragments, whereas PA28$\alpha\beta$ would operate as an exit channel for generated polyQ peptides, thereby preventing internal clogging of proteasomes (Figure 3, route 1). Although polyQ degradation is not improved, by flushing the polyQ peptides, the proteasome would at least remain active. The second possibility would be a sequential pathway involving two different composed proteasomes (Figure 3, route 2). When indeed the 26S proteasome would be unable to cleave the polyQ sequences present in mhtt, it would release the resulting pure polyQ fragments into the cellular environment [45]. However, PA28$\alpha\beta$ could bind to both sites of downstream 20S particles thereby opening both gates and enhance the proteasome activities towards polyQ peptide degradation [89]. The frequency of these PA28$\alpha\beta$ capped proteasomes seems to be low since it was shown that only 4% of the total proteasome pool in rabbit spleen had this composition [90], although this number differs dependent on the cell type used (e.g., 15% in HeLa cells) and can be increased by IFNγ [91]. To our knowledge, it is unknown whether these double-capped PA28$\alpha\beta$ proteasomes are present in brain tissues and whether they are increased during HD.

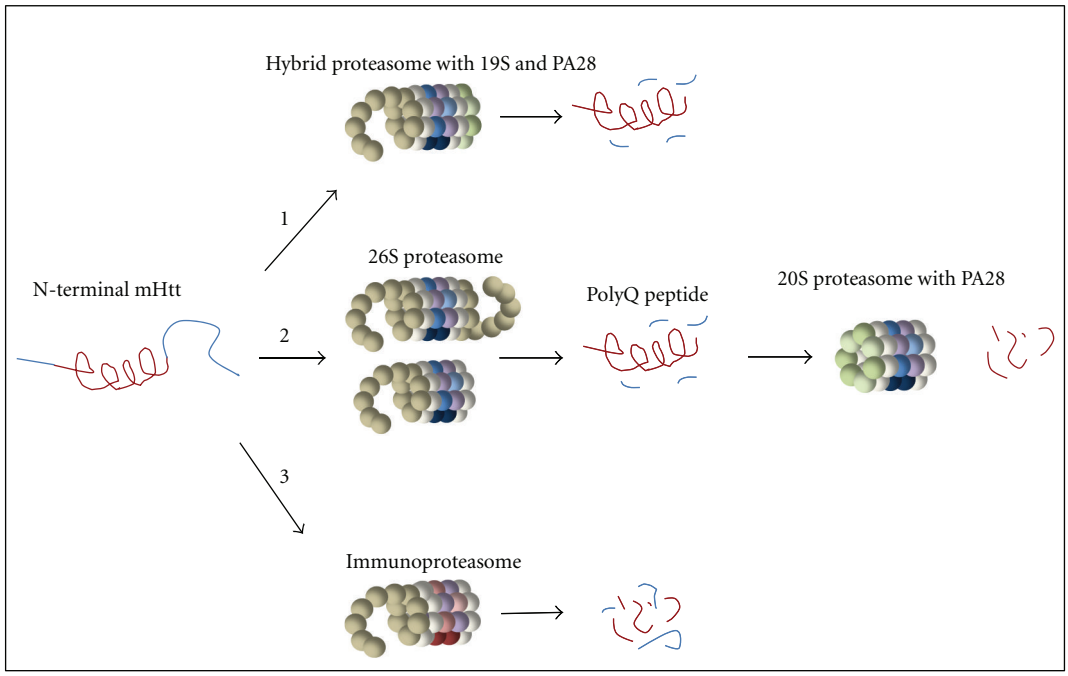

Figure 3: Potential proteasomal pathways for polyQ degradation. (1). Hybrid-proteasomes can recognize ubiquitinated proteins by the 19S cap and release the polyQ peptides faster due to an open gate conformation facilitated by PA28. (2) 26S proteasomes may be able to degrade mhtt but not the actual polyQ tracts. The released polyQ peptide could be targeted by PA28-capped proteasomes. (3) Changing the catalytic activity by immuno-subunit replacement might improve degradation preference for polyQ sequences.

3.3. The Proteasome Activator PA28γ. While PA28$\alpha\beta$ is mainly present in immune-related cells and generally absent from the brain, PA28γ could be a better candidate for proteasome activation due to its high expression in neurons [92]. Furthermore, the nuclear localization of PA28γ, in contrast to PA28$\alpha\beta$ which is mainly present in the cytoplasm, makes it an interesting proteasomal activator to target intranuclear mhtt. However, since expression of PA28γ inhibits the chymotrypsin-like activity, which seems to be the catalytic site important for cleaving polyQ peptides, it was speculated that downregulation of PA28γ would reduce the disease phenotype [65, 85, 93]. When R6/2 mice were crossed with PA28γ KO mice, no difference was seen in the behavioral phenotype nor in aggregate formation. In contrast, expression of PA28γ showed a protective role in an HD cellular model, but the observed increase in viability of cells that were exposed to stress conditions could also be due to the role of PA28γ as an antiapoptotic factor [94, 95]. Additionally, an intriguing mutation at lysine 188 in PA28γ altered the activation of the 20S proteasome due to destabilization of the PA28γ ring structure [85]. It is thought that due to this unstable conformation, the 20S core is differently structured thereby exposing all active sites with an increase of the proteasome activities similar to the activation changes induced by PA28$\alpha\beta$. *In vitro*, it has been demonstrated that the mutated activator PA28γ (K188E) increased activation towards polyQ fragments, since Q_{10}-peptides were degraded into fragments ranging between 1–9 glutamines [93]. This is in contrast to the earlier studies by Venkatraman and colleagues where it was shown that proteasomes could not cleave polyQ sequence [45]. Varying experimental conditions could explain these differences. As proposed for the PA28$\alpha\beta$, also PA28γ (K188E) could improve polyQ degradation in two different pathways, either as hybrid proteasomes or by improving cleavage of polyQ peptides released by upstream 26S proteasomes (Figure 3).

3.4. Proteasome Immunosubunits. Besides inducing PA28$\alpha\beta$, IFNγ also induces expression of the proteasome immuno-subunits LMP2 (β1i), LMP7 (β5i), and MECL-1 (β2i) which replace the constitutive catalytic subunits β1, β5, and β2, respectively. Incorporation of these newly synthesized subunits happens in de novo formed proteasomes within a time span that is four times faster than assembly of constitutive proteasomes [96, 97]. More important is the induced change in proteasome activity, as replacement of the constitutive subunits by immuno-subunits leads to down-regulation of the caspase-like activity and upregulation of the trypsin-like and chymotrypsin-like activities [98–100], although some discrepancies have been published on the induced alterations in proteasome activity and studies on activity changes induced by individual immuno-subunits also do not give conclusive results [76, 101, 102]. Interestingly, a similar increase in trypsin- and chymotrypsin-like activity of the proteasome is observed in human HD postmortem brains and in the HD94 mouse model, suggesting that immuno-proteasomes are induced in HD [63]. It is tempting to believe that this may reflect a protective response in order to degrade the accumulating polyQ fragments,

especially since immuno-proteasomes also appear to be important for cellular homeostasis [103, 104]. Another described consequence of IFNγ production is increased protein translation via the mTOR pathway, resulting in the generation of defective, unfolded, and oxidized proteins [105–107]. These defective ribosomal products (DRiPs) become polyubiquitinated and tend to form aggresome-like-induced structures (ALIS) as a cellular response to misfolded protein fragments in the cell. Therefore, simply inducing immuno-proteasomes by IFNγ to improve polyQ degradation might be counterintuitive, as the increase in DRIPs would only accelerate aggregation. However, in time the 19S cap dissociates from the 20S core and starts to forms complexes with the newly formed immuno-proteasome 20S particles [104]. These newly formed complexes appear to be better capable in preventing protein accumulations since mice deficient in immuno-subunits showed higher amounts of ALIS after IFNγ induction, indicating that immuno-proteasomes may be preferred to deal with the clearance of "dangerous" proteins and fragments. This is further supported by data showing that immuno-proteasomes and PA28$\alpha\beta$ are also involved in the increased degradation of oxidized proteins after treatment of cells with hydrogen peroxide (H_2O_2) [103]. The remaining question is whether we can use these immuno-proteasomes in order to clean up the polyQ fragments that induce toxicity in HD (Figure 3, route 3). The observed presence of immuno-subunits in HD94 mice has been shown to be a secondary effect due to inflammation [63, 108]. However, it remains unknown what would happen if immuno-proteasomes were present at an earlier stage of the disease.

4. Concluding Remarks

Since mhtt aggregates are mainly found in the nuclei of the affected neurons of human HD postmortem brain, it seems favorable to increase the degradation capacities in the nucleus. The UPS appears to be a robust mechanism in polyQ expressing cells, as it can recover after a temporary impairment [109]. Although it is clear that the proteasome is involved in the degradation of mhtt, the role of proteasomes remains contradictory. It is unknown whether proteasomes are the good guys as they can efficiently degrade nuclear mhtt fragments, or the bad guys for generating toxic, aggregation-prone polyQ peptides, or even the ugly guys when they become clogged and impaired by the polyQ fragments. In all cases, the modification of proteasome activity could stimulate them to improve clearance and prevent aggregation and toxicity of the polyQ fragments, not only in HD but also in related polyQ disorders. Introduction of different activators, exchanging the catalytic subunits or even using specific proteasome compounds that modulate proteasome activity might lead to improvement of the proteolytic cleavage of polyQ proteins. As proteins with an expanded polyQ stretch need to be soluble to enter the 20S core, a combination of proteasome activation and aggregate preventing compounds or chaperones could benefit the degradation process. It is known that particular heat-shock proteins can decrease aggregation rates in polyQ models, especially two members of the Hsp40 family (DNAJB6 and DNAJB8) are promising candidates [110–113]. Alternatively, chemical compounds and aggregation-interfering peptides like QBP1 could increase the solubility of mhtt to optimize its targeting for proteasomal degradation [20, 114]. Thus far, the general idea is that proteasomes are negatively affected in HD and have a great contribution to the disease course. The actual situation may be less grim, since recent data suggest that proteasomes are not impaired which makes it an interesting therapeutic target.

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Review Article

Ubiquitin-Mediated Regulation of Endocytosis by Proteins of the Arrestin Family

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In metazoans, proteins of the arrestin family are key players of G-protein-coupled receptors (GPCRS) signaling and trafficking. Following stimulation, activated receptors are phosphorylated, thus allowing the binding of arrestins and hence an "arrest" of receptor signaling. Arrestins act by uncoupling receptors from G proteins and contribute to the recruitment of endocytic proteins, such as clathrin, to direct receptor trafficking into the endocytic pathway. Arrestins also serve as adaptor proteins by promoting the recruitment of ubiquitin ligases and participate in the agonist-induced ubiquitylation of receptors, known to have impact on their subcellular localization and stability. Recently, the arrestin family has expanded following the discovery of arrestin-related proteins in other eukaryotes such as yeasts or fungi. Surprisingly, most of these proteins are also involved in the ubiquitylation and endocytosis of plasma membrane proteins, thus suggesting that the role of arrestins as ubiquitin ligase adaptors is at the core of these proteins' functions. Importantly, arrestins are themselves ubiquitylated, and this modification is crucial for their function. In this paper, we discuss recent data on the intricate connections between arrestins and the ubiquitin pathway in the control of endocytosis.

1. Introduction

The name of "arrestin" was initially given to a 48-kDa protein that was essential to "arrest" the signal following the photoexcitation of rhodopsin, a photoreceptor of the G-protein-coupled receptors (GPCRS) family expressed in rod and cone cells of the retina [1, 2]. A second isoform involved in the same process has later been identified; both of these proteins are now designated visual arrestins (or arrestin-1 and -4) (for review see [3]). A similar regulatory system was described for another GPCR, the β2-adrenergic receptor (β2-AR), which involves two other arrestins, named β-arrestin-1 and -2 (or arrestin-2 and -3, resp.) [4–6]. β-arrestins are ubiquitously expressed and were later found to regulate a large number of receptors in addition to β2-AR.

2. Arrestin-Mediated Regulation of GPCRs

2.1. Arrestin-Dependent Uncoupling of GPCRs from G-Proteins. Arrestins are key players in the regulation of GPCR signaling activity. Upon agonist stimulation, GPCRs undergo conformational changes leading to their association to heterotrimeric G proteins and subsequent activation, thereby triggering appropriate signal transduction pathways. Receptor desensitization is initiated after ligand binding through the phosphorylation of residues within their cytosolic loops by G-protein-coupled receptor kinases (GRKs). This modification allows arrestin docking to the GPCR, which in turn favors the uncoupling between the receptor and the G protein. Indeed, β-arrestins are cytosolic proteins that, in response to receptor stimulation, relocalize rapidly to

the plasma membrane [7]. Structural and structure-function studies of visual arrestins identified a phosphate-sensor domain in the polar core of the protein [8]. Intramolecular interactions between the C-terminal tail and the phosphate sensor region maintain the arrestin in an inactive state, and this interaction is disrupted upon binding to the phosphorylated receptor. This interaction is followed by a conformational change of the arrestin molecule and leads to a high-affinity receptor-binding state. Arrestin recruitment onto the phosphorylated receptor hinders its interaction with G protein, and consequently silences the activation of the GPCR-G protein-signaling module.

2.2. Arrestins and GPCR Endocytosis. Another crucial component of GPCR regulation operates at the level of their localization [9]. Endocytosis plays a major role in the modulation of GPCR signaling activity, and, again, this regulation involves β-arrestins [10]. Indeed, β-arrestins act as endocytic adaptor proteins that recruit components of the endocytosis machinery to promote GPCR internalization and/or degradation. β-arrestins interact with clathrin through a clathrin-binding motif [11–13] to promote GPCR association to clathrin-coated pits (CCPs). Deletion of the clathrin-binding site abrogates arrestin-promoted trafficking of the β2-AR [14]. Additionally, β-arrestins interact with the clathrin adaptor complex AP-2 upon GPCR binding [14–16], to promote clathrin-coat assembly and receptor targeting to CCPs [17]. In addition to clathrin, β-arrestins also bind to other components of the endocytic machinery such as the N-ethylmaleimide-sensitive fusion protein (NSF), the small G protein ARF6, and the phosphatidylinositol 4-phosphate 5 kinase PIP5 K Iα [18–20].

2.3. Arrestins as Signaling Scaffolds. Besides their functions in GPCR desensitization and trafficking, β-arrestins are also capable of generating their own signals by scaffolding signaling molecules, such as non-receptor tyrosine kinases of the Src family, or MAP (mitogen-activated protein) kinases (ERK1/2, c-Jun N-terminal kinase 3 JNK3) (reviewed in [21]). β-arrestins therefore mediate a second wave of signaling distinct from G-protein-dependent signaling.

2.4. Arrestins and Ubiquitin. β-arrestins were also shown to regulate the final fate of the receptor, by acting on the balance between receptor recycling to the plasma membrane, or its lysosomal degradation. The posttranslational modification of plasma membrane proteins, including receptors, by ubiquitin is known to affect their sorting along the endocytic pathway [22, 23]. β-arrestins have the ability to recruit ubiquitin ligases and promote receptor ubiquitylation, therefore acting as "adaptor" proteins [24]. Interestingly, a phylogenetic study has revealed that proteins of the arrestin family are present in all eukaryotes, except plants [25, 26]. A body of evidence (detailed later in this review, [27]) indicates that these arrestin-related proteins are also involved in the regulation of plasma membrane proteins trafficking by acting as ubiquitin ligase adaptors. Therefore, this function seems to be one of the most conserved features within the arrestin family [28, 29].

Both arrestins and arrestin-related proteins are themselves targets of ubiquitylation. This was discovered very early on for β-Arr2 in response to agonist stimulation [30]. Likewise, the fungal arrestin-related protein PalF was shown to be ubiquitylated in response to alkaline ambient pH, in a signal- and receptor- (PalH) dependent manner [31]. This ubiquitylation appeared crucial for the proper function of arrestins [24, 32–35], but the precise role of this modification is poorly understood. An additional layer of complexity has recently been added following the observations that β-arrestins interact with deubiquitylating enzymes that regulate their ubiquitylation status as well as receptor ubiquitylation and, consequently, their fate [35–37].

In this review, we will focus on the connections between arrestins and ubiquitin. We will detail the function of arrestins and arrestin-related proteins as ubiquitin ligase adaptor and discuss how arrestin functions could be regulated by ubiquitylation.

3. Arrestins as Ubiquitin Ligase Adaptors

3.1. Ubiquitin and Endocytic Protein Sorting. Studies in the last decades have shown that ubiquitin is a master regulator of endocytosis in eukaryotes. Early work performed in the yeast *Saccharomyces cerevisiae* demonstrated that ubiquitin is involved in the endocytosis of plasma membrane proteins, such as ABC (ATP-binding cassette) transporters [38], receptor[39, 40], or permeases [41]. The ubiquitylation of plasma membrane proteins appears to trigger their internalization and targeting to endosomes [42], although the existence of an ubiquitin-independent internalization mechanism is also documented [43]. In mammalian cells, the situation is more complex, as several internalization pathways exist in the cell with only some of them regulated by ubiquitin [44].

Initially, it has been proposed that ubiquitylated cargoes are recognized in yeast and mammals by the ubiquitin-binding motifs of various proteins involved in endocytosis, such as Eps15 (Ede1 in yeast) and Epsin (Eps15 interacting; Ent1 and Ent2 in yeast) which display UIM (ubiquitin-interacting motif) or UBA (ubiquitin-associated) domains [45, 46]. In addition, these endocytic proteins can also interact with phosphoinositides and clathrin, making them ideal candidates to coordinate ubiquitin recognition and cargo internalization. While such a function appears to be established in mammalian cells [47–49], recent data in yeast favor a more complex model, where ubiquitin-binding domains would play a more general role in protein interactions and the assembly of the endocytic network [50]. Noteworthy, in mammalian cells, endocytic adaptors are often ubiquitylated in response to extracellular stimuli, and this contributes greatly to the ubiquitin-based signaling triggered upon cell stimulation [23, 51–53].

A second major ubiquitin-dependent step in the endocytic pathway occurs at multivesicular bodies (MVBs) and is required for cargo delivery into lysosomes [54]. Cargo

ubiquitylation provides the crucial signal for entering into this pathway. A series of protein complexes, collectively named ESCRT (endosomal sorting complex required for transport) carry ubiquitin-binding domains and act in concert to allow the recognition and sorting of ubiquitylated cargoes into luminal vesicles of MVBs [55]. Therefore, lack of cargo ubiquitylation at this stage leads to a defective targeting to the lysosome, and, eventually, recycling [56].

In mammalian cells, initial studies showed that the ubiquitin conjugation system is important for the downregulation of the growth hormone receptor (GHR) [57]. Also, the study of the amiloride-sensitive epithelial sodium channel ENaC clearly established that its ubiquitylation regulates the channel's stability [58]. Subsequent work on ENaC, GHR, and many other receptors (such as EGFR, PDGFR, c-Met, TGF-βR, β2-AR) confirmed the critical function of ubiquitin in endocytosis in mammals [23, 59, 60]. However, where this ubiquitylation occurs in the cell (plasma membrane or endosomal compartments), and how ubiquitylation impacts on the target receptor's fate (internalization, progression through the endocytic pathway, or degradation) are still a matter of debate and seem to vary upon the receptor and the physiological situation considered [61]. Also, it should be noted that while ubiquitin-mediated endocytosis appears as the main pathway in yeast, ubiquitin-independent endocytosis is more represented in higher eukaryotes [44, 62].

3.2. The "Classic" β-Arr2/β2-AR Couple. A first evidence for the role of arrestins in receptor ubiquitylation came from a study by Shenoy and colleagues who observed that the β2-AR is ubiquitylated within 15 min of isoproterenol stimulation, ultimately leading to receptor degradation [30]. β2-AR ubiquitylation requires β-Arr2 and the ubiquitin ligase MDM2, which turned out to ubiquitylate β-Arr2 rather than β2-AR (see below) [30]. A mutant β2-AR lacking the ubiquitylation sites ($\beta 2 - AR^{K0}$) is normally internalized, but not degraded [30]. In contrast, a translational fusion of ubiquitin to the β2-AR, which mimics its constitutive ubiquitylation, is internalized similarly as the wild-type β2-AR, but is degraded more efficiently [24].

Therefore, ubiquitylation is a critical signal for β2-AR degradation upon stimulation. A similar implication of GPCR ubiquitylation in its degradation, but not in its internalization, was also reported in the case of CXCR4 [63]. The identity of the ubiquitin ligase responsible for β2-AR ubiquitylation was revealed more recently. Indeed, β-Arr2 was found to interact with the HECT-type (homologous to E6-AP C-terminus) ubiquitin ligase Nedd4 (discussed below in this paper). Nedd4 (neural precursor cell expressed developmentally downregulated protein 4) promotes β2-AR ubiquitylation at endosomes, leading to its lysosomal targeting [24].

Once internalized, GPCRs can also escape degradation and recycle back to the plasma membrane in a functional state to mediate further signaling. Because GPCR ubiquitylation appears to trigger its degradation, deubiquitylation could regulate GPCR recycling to the plasma membrane. Indeed, two deubiquitylating enzymes named USP33 and USP20 regulate β2-AR deubiquitylation, recycling and resensitization [35, 37]. USP33 was first identified as a β-arrestin interactant, thus suggesting that β-Arr2 could be involved in USP33 recruitment to β2-AR [35]. However, USP33 was found to interact with β2-AR even before agonist stimulation, that is, when β-Arr2 is not yet translocated to the plasma membrane [37]. In fact, USP33 appears to be transferred from agonist-activated β2-AR to β-Arr2, thus triggering its deubiquitylation and dissociation from the receptor, once internalized. Reassociation of USP33 with β2-AR in endosomal compartments would regulate its deubiquitylation and recycling to the plasma membrane. Thus, the association and dissociation of β-Arr2 from β2-AR may coordinate the ubiquitin conjugating/deconjugating activities towards β2-AR to tune the balance between receptor degradation and recycling. This positions the ubiquitin ligase adaptor function of β-Arr2 as a key regulator of GPCR signaling.

3.3. β-Arrestins as Ubiquitin Ligase Adaptors: Other Examples. The function of β-arrestins as ubiquitin ligase adaptors is not restricted to β2-AR. Additional studies identified β-arrestins as ubiquitin ligase adaptors for non-GPCR proteins: β-Arr1, as β-Arr2, acts as an adaptor for ubiquitin ligases of the Nedd4 family such as Itch/AIP4 (Atrophin-1-interacting protein 4) for ubiquitylation of the TRPV4 (transient receptor potential) channel [64], and Nedd4 for that of the Na^+/H^+ exchanger 1 (NHE1) [65]. In the latter case, however, and in contrast to the situation described for the β2-AR, cargo ubiquitylation is required for its internalization. Because β-arrestins interact with both ubiquitin ligase and clathrin (see above), they may then act at two levels: first, for cargo ubiquitylation, which could recruit Eps15/Epsin endocytic adaptors, and, second, to assist the latter in the recruitment of a clathrin coat.

The contribution of β-arrestins to the trafficking of another classical GPCR, the chemokine receptor, CXCR4, was also studied. Early reports had shown that the ligand-induced ubiquitylation of CXCR4 by the Nedd4-like ubiquitin ligase AIP4 is required for its lysosomal sorting [63, 66]. β-Arr1 interacts with AIP4 at endosomes, and knockdown experiments revealed that β-Arr1 is an important player in CXCR4 degradation but, surprisingly, is not required for its ubiquitylation [67]. Instead, CXCR4 is phosphorylated at the plasma membrane after ligand binding, which allows the direct recruitment of the ubiquitin ligase AIP4 via its WW domains, and hence CXCR4 ubiquitylation [68]. β-Arr1 was later found to interact with the ESCRT-0 complex and to direct the ubiquitylation of one of its components, HRS (hepatocyte growth factor-regulated tyrosine kinase substrate) in a CXCR4-dependent manner [69].

Interestingly, β-arrestins appear to act primarily as adaptors for ubiquitin ligase of the Nedd4 family. These enzymes display WW domains that can interact with specific proline-rich motifs (usually, a [L/P]PxY sequence). Although this motif is sometimes present on the targeted substrates, as in the case of ENaC [70], in most cases this interaction

motif is present on an adaptor protein in charge of substrate recognition [71]. However, no PPxY motif has been found in β-arrestins, and polyproline regions are not involved in Nedd4 interaction [24]. In addition, Nedd4 recruitment to β2-AR was not affected by mutations in Nedd4 WW domains. This indicates that this interaction involves a noncanonical binding of β-Arr2 to Nedd4, for which the molecular determinants remain to be addressed.

In some cases, β-arrestins act as adaptors for ubiquitin ligase which do not belong to the Nedd4 family. β-Arr1 was proposed to act as an adaptor for the RING (Really interesting new gene) ubiquitin ligase Mdm2 to mediate insulin-like growth factor I (IGF-1) receptor ubiquitylation and downregulation [72, 73]. A similar role was appointed to β-Arr2 for the ubiquitylation of the androgen receptor [74]. Again, the molecular basis of this interaction awaits further investigations.

4. Arrestin-Related Proteins: New Players in the Field

Visual and β-arrestins share a similar structure, with an arrestin fold in their N-terminal domains and a C-terminal tail [75–80]. It was proposed that visual and β-arrestins actually originate from an ancestral arrestin family from which they diverged relatively recently [25]. This ancestral family would also have given rise to proteins whose expression is not limited to metazoans: members of the Vps26 family, which display an arrestin-like fold [81, 82], as well as arrestin-related proteins (also coined α-arrestins) [25]. Indeed, proteins displaying sequence homologies to arrestins were first identified in the filamentous fungus *Aspergillus nidulans*, named CreD [83] and PalF [31], and more recent work in yeast allowed to identify additional members of this protein family renamed "ART" (arrestin-related trafficking adaptors) [34, 84, 85] that will be discussed later in this paper. In human, the ART family is composed of six members, named arrestin-domain containing 1–5 (ARRDC1–5) and TXNIP (Thioredoxin-interacting protein) (Figure 1). Therefore, arrestin-related proteins are expressed in all eukaryotes, except plants, which interestingly do not harbor Nedd4-like genes either [25].

4.1. Arrestin-Related Proteins as Endocytic Adaptors. A main difference between visual/β-arrestins and arrestin-related proteins is that the latter possess PPxY motifs (Figure 1). In agreement with the reported function of these motifs (see above), many studies have documented the ability of yeast arrestin-related proteins to interact with the only Nedd4-like ubiquitin ligase in *S. cerevisiae*, named Rsp5 [33, 34, 85–90]. Rsp5 is critical for ubiquitin-dependent intracellular trafficking pathways, such as endocytosis and MVB sorting [91]. However, most of the transporters lack PPxY motifs, and until recently, the molecular basis for the interaction between Rsp5 and transporters was unknown. It has become clear that yeast arrestin-related proteins fulfill this function, by acting as Rsp5 adaptors to mediate ubiquitylation and subsequent endocytosis of transporters [33, 34, 84, 85, 90]. Using a chemical-genetic screen, Emr and colleagues have identified Ldb19/Art1 as a regulator of the endocytosis of Can1, an arginine transporter [34]. The function of Ldb19/Art1 was also extended to the endocytosis of other amino acid transporters. In a parallel study, Nikko et al. showed that two other arrestin-related proteins, named Ecm21/Art2 and Csr2/Art8, are specifically involved in the downregulation of the manganese transporter Smf1 [85]. Altogether, around 10 arrestin-related proteins were identified in yeast [34, 84, 85], and gathered in a family referred to as "ART" (arrestin-related trafficking adaptors).

Contrary to the situation in mammalian cells, where β-arrestins mainly act at a late step in cargo sorting, studies in yeast suggested a role for arrestin-related proteins in cargo internalization at the plasma membrane [27]. Indeed, several yeast ARTs are involved in the signal-induced internalization of transporters in response to specific environmental signals [33, 34, 84, 85, 90]. In addition, Art1 relocalizes to the plasma membrane in response to the signal that induces amino acid transporter endocytosis [34, 92].

However, as for β-arrestins, the situation is probably more complex, and the role of ARTs in endocytosis may not be restricted to the plasma membrane. In the course of their study of the high-affinity iron-uptake protein complex Fet3/Ftr1 in yeast, Burd and colleagues documented an example of ubiquitin-independent internalization [43]. The results show that a nonubiquitylatable form of Fet3/Ftr1 can still be internalized but is constitutively recycled back to the plasma membrane, leading to an apparent defect in internalization. Although the involvement of an arrestin remains to be determined, it strongly suggests that in this system, cargo ubiquitylation by Rsp5 is required at endosomal compartments, rather than at the plasma membrane. In addition, two yeast arrestin-related proteins, Aly1/Art6 and Aly2/Art3, have been shown to localize to intracellular compartments and to control the trafficking of the general amino-acids transporter Gap1 between trans-Golgi and endosomes [93]. Consistent with these findings, Aly1 and Aly2 interact with both clathrin and Golgi-specific clathrin adaptor complex AP-1, thus suggesting that arrestin related proteins, as β-arrestins, promote clathrin-coat assembly and cargo targeting to clathrin-coated vesicles. Therefore, futures studies will be necessary to precise where yeast arrestin-related proteins act on cargo trafficking. Regarding their intracellular localizations, we can already hypothesize several modes of action within the ART family of proteins.

Like their yeast homologs, several human ARRDC proteins are able to interact with ubiquitin ligases of the Nedd4 family [94–97]. Among those, ARRDC3 was isolated in a screen designed to identify proteins involved in β2-AR ubiquitylation and degradation after agonist treatment [95]. ARRDC3, as β-Arr2 [24], was shown to bridge the interaction between Nedd4 and β2-AR, leading to the intriguing possibility that arrestin-related proteins might coordinate, together with β-arrestins, receptor ubiquitylation and degradation. Because both classes of arrestins have the ability to dimerize [94, 98], this raises the possibility of potential heterooligomers between arrestin and arrestin-related proteins that

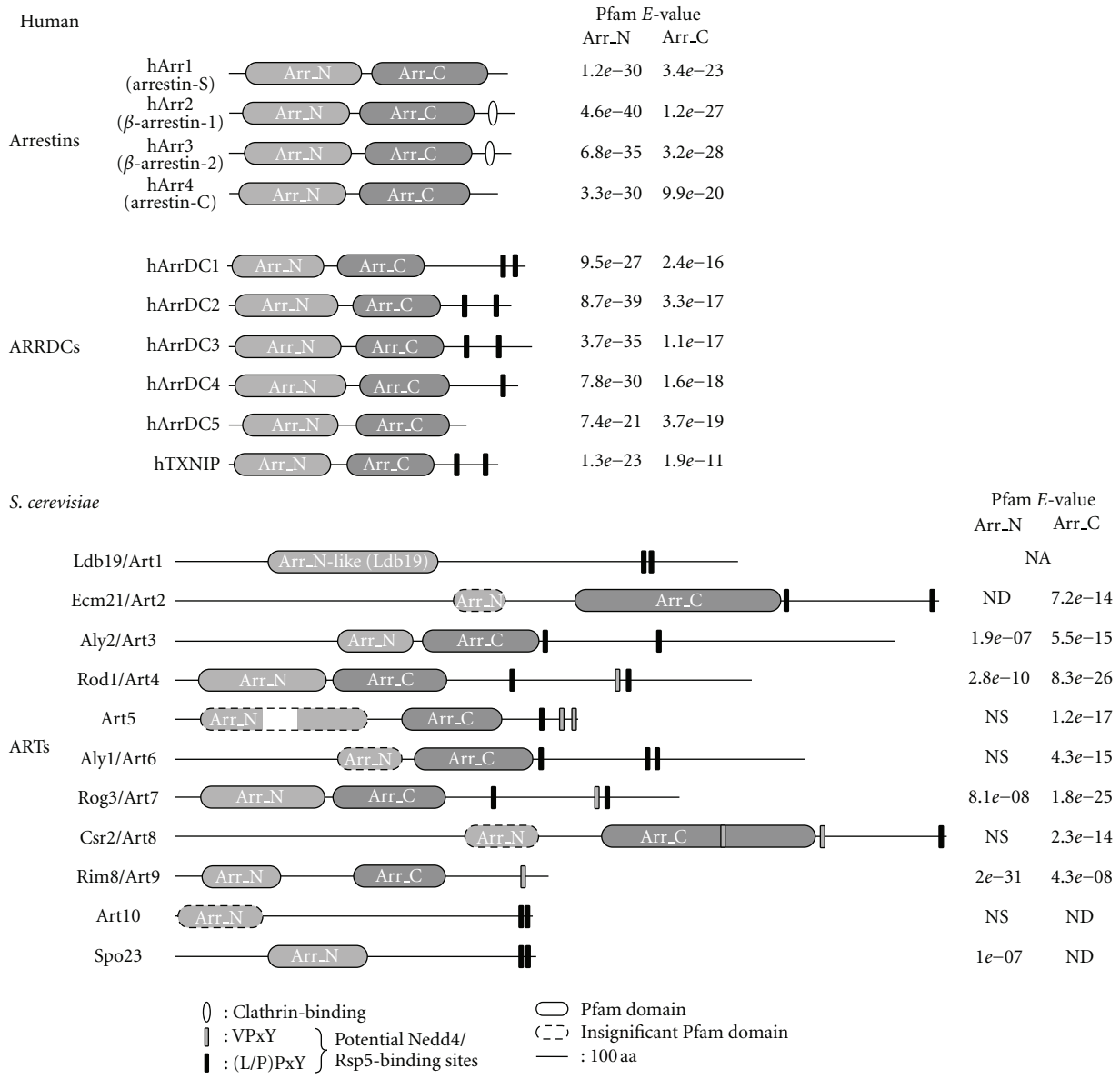

Figure 1: Schematic representation of the domain organization of human arrestins and arrestin-domain containing (ARRDC) proteins, and yeast ARTs (arrestin-related trafficking adaptors). Domains detected by Pfam 26.0 (http://pfam.sanger.ac.uk/) are shown and correspond to the following Pfam-A accessions: Arr_N: PF00339, Arr_C: PF02752, Arr_N-like (Ldb19): PF13002, along with the corresponding E-values for each domain (NA: not applicable; ND: not detected; NS: not significant). A putative Arr_N domain in Ecm21/Art2 was identified by alignment with the presumed Arr_N domain of Csr2/Art8. Clathrin-binding sites are depicted on β-arrestins; potential binding sites for ubiquitin ligases of the Nedd4 family are also indicated on arrestin-related proteins.

could reveal a complementary role between arrestin classes.

The basic function of arrestin-related proteins as ubiquitin ligase adaptor therefore seems strongly conserved. Of note, a role for ARRDC3 in the degradation of a cell surface adhesion molecule, integrin β4, was also pointed out, but its role as ubiquitin ligase adaptor in this context has not been investigated [99]. Future studies will indubitably unravel new connections between ubiquitin and arrestin-related proteins.

4.2. Other Functions of Arrestin-Related Proteins: An ESCRT Connection. As previously mentioned, arrestin-related proteins were initially identified in *A. nidulans* and named CreD [83] and PalF [31]. Interestingly, in both cases, a connection with the ubiquitin pathway was established. CreD was shown to interact physically with the Nedd4 homologue in *A. nidulans*, HulA, whereas PalF was found to be ubiquitylated in vivo. PalF, a protein involved in the ambient pH signaling in fungi, binds to the seven-transmembrane and putative

pH sensor, PalH. This pointed out to many similarities between mammalian β-arrestins and this arrestin-related protein [31].

As in *A. nidulans*, the yeast PalF homologue Rim8/Art9 is essential for the proteolytic activation of the pH-responsive transcription factor, Rim101, in response to neutral-alkaline pH [100]. Interestingly, there is an intricate connection between the ESCRT machinery, involved in ubiquitin-dependent cargo sorting at the MVB, and this signaling pathway [100–102]. The ART Rim8/Art9 is central to the coordination of the ESCRT machinery and the pH-signaling pathway, as it interacts with both the putative pH sensor Rim21 and the ESCRT-I subunit Vps23 [89]. ESCRT appears to provide a platform for recruitment of a protein complex containing the ESCRT-III binding protein and ALIX homologue Rim20, that enables the proteolytic activation of the Rim101 transcription factor in response to the pH signal. Although initial studies suggested that this process takes place at the endosomal membrane [103], subsequent work supported the idea that arrestin-mediated recruitment of ESCRT in the fungal ambient pH signaling pathway may occur at the plasma membrane [89, 104]. Similarly, some of the human ARRDCs interact with the Vps23 homologue TSG101 or the ESCRT-associated protein ALIX [90, 96]. In particular, ARRDC1-mediated recruitment of ESCRT appears to drive the formation of microvesicles at the plasma membrane that may be involved in intercellular communication [94, 105]. Interestingly, this situation is reminiscent of the budding step of different enveloped RNA viruses, which recruit ESCRT components through similar interactions to promote membrane scission and subsequent viral particle release [106]. Accordingly, overexpression of several ARRDCs inhibits murine leukemia virus (MLV) viral particle release in a PPxY-specific way [96]. Therefore, ARRDCs may act as adaptors between Nedd4-like enzymes and the ESCRT machinery, in viral budding.

Finally, the connection between arrestin and the ESCRT machinery may not be restricted to arrestin-related proteins. Indeed, and as previously mentioned, β-Arr1 was found to interact with STAM-1 (signal-transducing adaptor molecule), a component of ESCRT-0, to regulate endosomal sorting of CXCR4 [69].

5. Regulation of Arrestin Function by Ubiquitin

5.1. Regulation of β-Arrestins by Ubiquitylation. Arrestins are specifically recruited to the cargoes following agonist stimulation (receptor) or in response to the presence of the substrate (transporter, channel), thus suggesting that they are regulated to mediate an adapted response of the cell to extracellular changes.

As mentioned previously, Shenoy and colleagues showed in a seminal article that β-Arr2 is itself ubiquitylated in response to agonist treatment [30]. Ubiquitylation of β-Arr2, in contrast to that of β2-AR, does not require Nedd4, but an ubiquitin ligase of the RING family, Mdm2. As this modification occurred upon stimulation, this suggested a role for β-Arr2 ubiquitylation in β-AR trafficking. Indeed, Mdm2 knockdown caused a defect in β2-AR internalization. Thus, β-Arr2 ubiquitylation appears to play a key role in GPCR trafficking, and several lines of evidences support this idea.

To address directly the importance of this posttranslational modification and to avoid potential indirect effects of the knockdown of the Mdm2 ubiquitin ligase on β2-AR trafficking, studies were performed using both a nonubiquitylatable mutant form of β-Arr2 ($\beta - Arr2^{0K}$) and a translational fusion of ubiquitin to β-Arr2 (β-Arr2-Ub) [107]. These experiments showed that $\beta - Arr2^{0K}$ recruitment to the plasma membrane was only transient and unable to trigger internalization of β2-AR. On the opposite, translational fusion of ubiquitin to β-Arr2 led to its co-trafficking with β2-AR into endosomal compartments [107]. Previous observations had classified GPCRs in two classes (A and B), based on the interaction pattern between receptor and β-arrestin. Interaction of β-arrestin with class A receptors (e.g., β2-AR) only takes place at the plasma membrane, while its interaction with class B receptors (e.g., angiotensin II type 1a receptor: AT1aR, or vasopressin V2 receptor: V2R) is more stable and persists even after receptor internalization [108]. Interestingly, the increased stability of the interaction between class B receptors and β-arrestin correlates with a sustained β-arrestin ubiquitylation, which is not observed with class A receptors [36]. Indeed, even if $\beta - Arr2^{K0}$ is able to interact with the receptor *in vitro*, this interaction is weaker than that displayed with the wild type form *in vivo*. On the opposite, translational fusion of ubiquitin to β-Arr2 displays a stronger binding than wild type β-Arr2 [107]. β-Arr2 ubiquitylation therefore appears to reinforce the interaction with β2-AR.

Because β-Arr2 is capable of interacting with the endocytic machinery, such as clathrin or clathrin adaptors, the failure of $\beta - Arr2^{K0}$ to promote β2-AR internalization could originate from an impaired interaction with these components. Indeed, $\beta - Arr2^{K0}$ exhibits a weaker interaction with clathrin than the wild-type form [107]. While clathrin is not known to interact with ubiquitin, β-Arr2 ubiquitylation might stabilize the interaction with clathrin through other components of the endocytic machinery such as Eps15/epsin proteins that are able to bind both ubiquitin and clathrin.

β-Arr2 ubiquitylation was also shown to affect its scaffolding function for signaling proteins. The amplitude of β-arrestin-mediated activation of ERK (extracellular signal-regulated kinase) correlates with the β-Arr2 ubiquitylation status. Although β-Arr2 ubiquitylation was not required for its interaction with MAP kinases (such as c-Raf and ERK), translational fusion of ubiquitin to β-Arr2 led to an increased level of ERK activity in endosome localized receptor complexes (signalosomes). Consistent with these findings, β-arrestin ubiquitylation promote its association with membrane. Again, ubiquitylation appears to function in stabilizing the β-arrestin-mediated interaction between the receptor and signaling proteins [107].

Finally, arrestins undergo conformational changes upon binding to activated receptors [109]. Ubiquitin modification

could therefore contribute to the proper rearrangement of the β-arrestin structure, leading to optimal interactions with its partners, and this awaits further investigations.

5.2. Regulation of Arrestin-Related Protein by Ubiquitylation. Many arrestin-related proteins have also been reported as substrates for ubiquitylation, both in fungi and human [31, 33, 34, 89, 90, 94, 96, 97, 110]. Ubiquitylation of these proteins, in contrast to that of β-arrestins, is triggered by ubiquitin ligases of the Nedd4 family. Therefore, arrestin-related proteins are adaptors as well as targets of the same ubiquitin ligases.

The yeast arrestin-related protein Ldb19/Art1 is required for the endocytosis of amino acid permeases, such as the arginine transporter, Can1. Failure to endocytose Can1 leads to sensitivity of the cells to canavanine, a toxic analog of arginine. A nonubiquitylatable mutant of Ldb19/Art1 cannot grow on this drug, suggesting that Can1 remains at the plasma and therefore that Art1 is not functional [34]. The importance of ubiquitylation for ART function was also demonstrated for Rod1/Art4, involved in the glucose-induced endocytosis of carbon sources transporters [33, 84]. Rod1/Art4 is ubiquitylated in response to glucose exposure and a nonubiquitylatable mutant is unable to promote the endocytosis of the lactate transporter, Jen1, following glucose treatment [33]. Altogether, these data indicate that ART ubiquitylation is crucial for their function in endocytosis. Human arrestin-related protein ARRDC3 was isolated in a screen designed to identify genes involved in β2-AR degradation, and acts as a Nedd4 adaptor for β2-AR ubiquitylation [95]. While ARRDC3 ubiquitylation has not yet been observed, ARRDC1 and TXNIP were shown to be ubiquitylated by ubiquitin ligases of the Nedd4 family [90, 96, 97]. Thus, it is tempting to speculate that the same regulation applies in fungi and human.

The ubiquitylation of the arrestin-related protein PalF in *A. nidulans* is triggered in a signal-(alkaline pH) and receptor-(PalH) dependent manner [31]. PalF ubiquitylation appears as a major determinant of its activity, since the translational fusion of ubiquitin to PalF leads to a constitutive activation of the pathway [110]. The yeast PalF homologue Rim8/Art9 was shown to be monoubiquitylated [89]. Monoubiquitylation of Rim8/Art9 occurs on a lysine residue in its C-terminus and, as for all other ARTs described to date in yeast, is performed by Rsp5, which binds to a PxY motif near the ubiquitylation site. This monoubiquitylated residue, together with a SxP motif, contributes to the interaction of Rim8/Art9 with the ESCRT-I subunit Vps23 via its ubiquitin-binding domain, UEV (ubiquitin E2 variant) [89]. Interestingly, Vps23 binding appears to control the levels of monoubiquitylated Rim8/Art9, thus suggesting that this interaction either promotes Rim8 ubiquitylation or prevents its further polyubiquitylation and possibly its degradation. Interaction of human ARRDC1 with the Vps23 homologue Tsg101 was shown to be mediated by a PSAP motif which, like the SxP motif in Rim8, is located at the protein C-terminus [94, 96]. In addition, it was proposed that the ubiquitylation of ARRDC1 is important for its function [94]. However, this is based on results obtained upon depletion of the corresponding Nedd4-like ligase WWP1, which in principle could also impair a potential adaptor function and may have off-target effects. Therefore, the identification and mutation of the ubiquitylation sites will be critical to address this question.

5.3. Dynamic Regulation of Arrestin Ubiquitylation. Phosphorylation-Dependent Ubiquitylation? Because ubiquitin ligases target a large number of proteins in the cell, their activity toward a given substrate is usually indirectly regulated through substrate accessibility, either by the use of adaptor proteins, or by post-translational modification of the substrate, such as phosphorylation [111].

Interestingly, cytosolic β-arrestins are constitutively phosphorylated, and undergo dephosphorylation upon binding to the activated receptor. β-Arr1 dephosphorylation is required for β2-AR internalization, but not for its desensitization [112]. Indeed, a β-Arr1 mutant mimicking constitutive phosphorylation displays a weaker interaction with clathrin but an unaltered β2-AR binding [112]. Similar data were reported for β-Arr2, and the phosphorylation site was localized near the clathrin and AP-2 binding motifs, thus providing an explanation as to why β-Arr2 phosphorylation regulates the interaction with clathrin/AP-2 [113]. Additionally, the phosphorylation of the major visual arrestin in *Drosophila* (Arr2) also regulates its interaction with clathrin [114].

Importantly, once the receptor is internalized, β-Arr1 is rephosphorylated. These dynamic phosphorylation/dephosphorylation events suggest the involvement of kinases and phosphatases whose activation is coordinated in response to agonist exposure. Interestingly, β-Arr1 is phosphorylated *in vitro* by ERK kinases and accordingly, the modulation of ERK activity *in vivo* affects β-Arr1 phosphorylation, thus providing an inhibitory feedback control of its function [115].

Although β-arrestins are both dephosphorylated and ubiquitylated upon receptor binding, an eventual relationship between these two modifications remained to be addressed. Such a link has been described for the yeast arrestin-related protein Rod1/Art4, involved in the glucose-induced endocytosis of carbon sources transporters [33]. As for β-arrestins, Rod1/Art4 dephosphorylation and ubiquitylation occurs in response to an external signal-in this case, glucose. The yeast homologue of AMPK (5′-AMP-activated protein kinase), Snf1, and its counteracting phosphatase PP1 (protein phosphatase 1) control the phosphorylation status of Art4/Rod1 in response to glucose availability. Therefore, in the absence of glucose, Art4/Rod1 is phosphorylated and endocytosis is inhibited. This inhibitory effect results from the ability of phosphorylated Art4/Rod1 to bind 14-3-3 proteins, thereby hindering its ubiquitylation by Rsp5 and hence preventing its activation [33].

Interestingly, phosphorylation of another yeast arrestin-related protein, Ldb19/Art1, also regulates its function. A recent study indicated that Ldb19/Art1 is subject to

phosphoinhibition through the action of the TOR (target of rapamycin) effector and protein kinase Npr1, thus allowing cells to regulate amino acid transporter endocytosis in response to the nitrogen status [92]. While the overall phospho-inhibition mechanism recalls that of Rod1/Art4, Ldb19/Art1 ubiquitylation is uncoupled from its phosphorylation, suggesting a different regulatory mechanism [34]. The identification of Npr1-dependent phosphorylation sites on Ldb19/Art1 allowed generating a nonphosphorylatable mutant form of the protein. Interestingly, this mutant fails to be translocated at the plasma membrane upon stimulation, which likely explains why transporters endocytosis is impaired [92]. Further work will be needed to understand the molecular mechanism of this phosphorylation-dependent inhibition.

Other examples of arrestin-related proteins that are subjected to phosphorylation which include *A. nidulans* PalF, involved in ambient pH sensing, although the regulatory mechanism appears to be different since PalF undergoes phosphorylation instead of dephosphorylation, in response to the ambient pH signal [31]. The same result has been observed in the pathogenic yeast *Candida albicans*, where the PalF homologue Rim8 is also phosphorylated in response to neutral-alkaline pH [116]. Although phosphorylation of Rim8/Art9 in baker's yeast has not been reported, its ubiquitylation does not appear to be regulated by ambient pH, in contrast to that of PalF, which is induced by alkaline pH [89]. The apparent lack of regulation of Rim8/Art9 ubiquitylation is consistent with its role in Vps23 binding, which appears to occur even in nonstimulated conditions. Thus, the pH-dependent regulation of PalF ubiquitylation in *A. nidulans* may reflect an additional level of regulation in this organism.

From these studies, it emerges that arrestin-related proteins are often modified posttranslationally in response to stimulation, either by phosphorylation, ubiquitylation, or both. A crosstalk between phosphorylation and ubiquitylation has been evidenced. How these modifications operate to coordinate arrestin function is unknown, and this provides new avenues for research in this field.

Ubiquitylation and deubiquitylation. Ubiquitylation, akin to phosphorylation, is a reversible process. Therefore, deubiquitylation appeared as a possible mechanism for regulation of arrestin function. In support of this idea, the transient β-Arr2 ubiquitylation associated to class A receptor suggested that deubiquitylation occurs rapidly after agonist stimulation. Indeed, the ubiquitin-specific protease 33 (USP33) was shown to deubiquitylate β-Arr2 following β2-AR binding [35]. USP33 knock-down led to an increase in β-Arr2 ubiquitylation. This was accompanied by a stronger interaction with the receptor, and a prolonged β-Arr2-dependent MAP kinase signaling. These findings are consistent with the phenotypes of cells expressing a translational fusion of ubiquitin to β-Arr2 (see above) and provide an additional mechanism for the regulation of arrestin function. Strikingly, recruitment of USP33 to β-Arr2 depends on the receptor class, in agreement with previous finding that receptor class determines the kinetics of β-Arr2 deubiquitylation [36]. In addition, receptors belonging to the same class can target different lysine residues on β-Arr2 for ubiquitylation [32]. Therefore, binding of β-Arr2 to different receptors may trigger distinct conformational change that could modulate both ubiquitylation sites accessibility and association with deubiquitylating enzymes, hence leading to a different functional output—in full support of the concept of an "ubiquitylation code."

6. Concluding Remarks

In this paper, we emphasized the many relationships between ubiquitin metabolism and arrestin biology. Many proteins of the arrestin family act as ubiquitin ligase adaptors and are required for ubiquitylation of endocytic cargo. In particular, recent data obtained on arrestin-related proteins have pointed out several features shared with β-arrestins, such as the intimate connection existing between these proteins and ubiquitin ligases of the Nedd4 family. In addition, ubiquitin also regulates arrestin function in a yet undefined way, which is now critical to understand. However, the emergence of new regulatory mechanisms involving a now expanded family of arrestin proteins, combined with the multiplicity of model organisms, is likely to favor the rapid evolution of concepts in arrestin biology.

Abbreviations

AIP4: Atrophin-interacting protein 4
AP-1/AP-2: Clathrin adaptor protein
ARRDC: Arrestin-domain Containing
ART: Arrestin-related trafficking adaptors
β-Arr: β-arrestin
β2-AR: β2-adrenergic receptor
ERK: Extracellular signal-regulated Kinase
ESCRT: Endosomal sorting complex required for transport
GPCRS: G-protein-coupled receptors.
MAP: Mitogen-activated protein
MVBs: Multivesicular bodies
Nedd4: Neural precursor cell expressed developmentally downregulated protein 4
RING: Really interesting new gene
TXNIP: Thioredoxin-interacting protein.

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Review Article

Strategies to Identify Recognition Signals and Targets of SUMOylation

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SUMOylation contributes to the regulation of many essential cellular factors. Diverse techniques have been used to explore the functional consequences of protein SUMOylation. Most approaches consider the identification of sequences on substrates, adaptors, or receptors regulating the SUMO conjugation, recognition, or deconjugation. The large majority of the studied SUMOylated proteins contain the sequence [IVL]KxE. SUMOylated proteins are recognized by at least 3 types of hydrophobic SUMO-interacting motifs (SIMs) that contribute to coordinate SUMO-dependent functions. Typically, SIMs are constituted by a hydrophobic core flanked by one or two clusters of negatively charged amino acid residues. Multiple SIMs can integrate SUMO binding domains (SBDs), optimizing binding, and control over SUMO-dependent processes. Here, we present a survey of the methodologies used to study SUMO-regulated functions and provide guidelines for the identification of *cis* and *trans* sequences controlling SUMOylation. Furthermore, an integrative analysis of known and putative SUMO substrates illustrates an updated landscape of several SUMO-regulated events. The strategies and analysis presented here should contribute to the understanding of SUMO-controlled functions and provide rational approach to identify biomarkers or choose possible targets for intervention in processes where SUMOylation plays a critical role.

1. Introduction

Posttranslational modifications (PTMs) by members of the ubiquitin family are covalent events that promote radical changes in the properties of modified proteins. Among all ubiquitin-like molecules, a particular attention has been given to the modification by SUMO (Small Ubiquitin MOdifier) also known as Sentrin. SUMOylation plays critical roles in a variety of cellular processes, including transcription, cellular localization, DNA repair, and cell cycle progression [1–3]. In mammals, there are four reported SUMO paralogues named SUMO-1 to SUMO-4 (Figure 1). SUMO-2 and SUMO-3, often referred as SUMO-2/-3, show a high degree of similarity and are distinct from SUMO-1 (approx., 50% similarity). SUMO-4 shows 87% similarity to SUMO-2/-3. However, SUMO-4, in contrast to SUMO-1, SUMO-2, and SUMO-3, seems to be insensitive to SUMO-specific proteases due to the presence of Pro-90. This may impair the processing of SUMO-4 to a mature form and its conjugation to substrates [3, 4]. Mass-spectrometric proof for the existence of conjugated SUMO-4 at the endogenous level is currently still missing, therefore, its relevance is still under debate. In mammals, SUMOylation is executed through a thiol-ester cascade of reactions mediated by the heterodimeric SUMO activating enzyme SEA1/SEA2 (in yeast Aos1/Uba2) or E1, the SUMO conjugating enzyme Ubc9 or E2 and a SUMO-E3-ligase specific for each target protein. Several families of SUMO E3s have been reported whose action appears to be in a dynamic equilibrium with

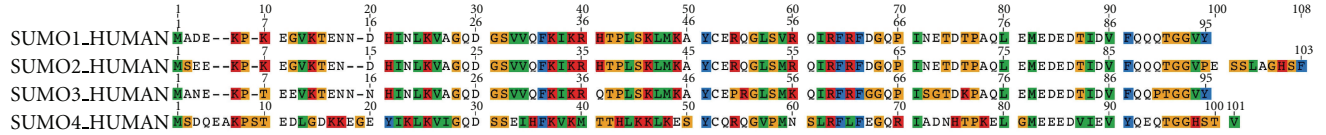

Figure 1: Sequence alignment of Homo sapiens SUMO-1 to SUMO-4. UNIPROT sequences shown are SUMO1 (P63165), SUMO2 (P61956), SUMO3 (P55854), and SUMO4 (Q6EEV6). The alignment is CLUSTAL colored using the software Geneious v4.8.5 (available from http://www.geneious.com/).

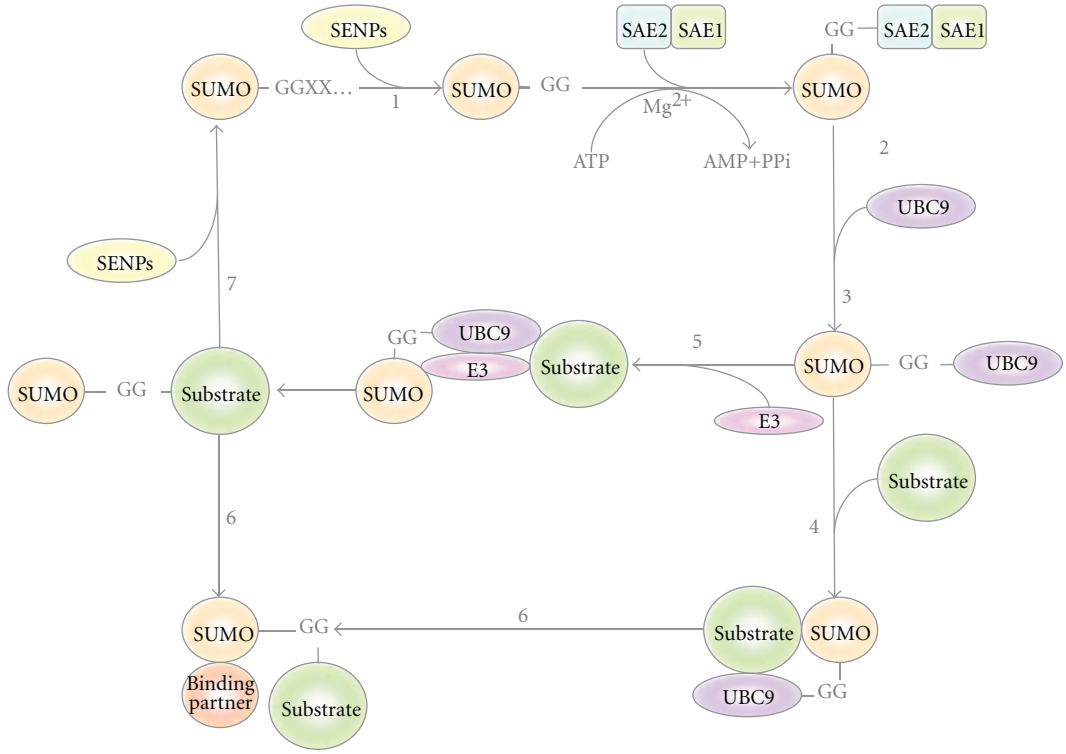

Figure 2: The SUMO conjugation pathway. The immature form of the Small Ubiquitin MOdifier (SUMO) undergoes processing by Ubiquitin-like protein-specific protease (Ulp) and SUMO/Sentrin-specific proteases (/SENPs) to generate its mature form (step 1), revealing a carboxy-terminal Gly-Gly motif. SUMO is then adenylated by the Aos1/Uba2 also named SAE1/SAE2 complex in an ATP·Mg^{2+}-dependent reaction (step 2). Following activation, SUMO is transferred to the catalytic Cys of the E2 conjugating enzyme (UBC9) (step 3), which can then catalyze SUMO conjugation to a substrate containing the SUMO consensus motifs (ΨK x E) in an E3 ligase-independent (step 4). SUMO E3 ligases can also facilitate SUMO transfer to the substrate proteins (step 5). Substrates modified by SUMO can interact with SUMO-binding proteins through their SUMO-interacting motifs (SIMs) (step 6). SUMO-deconjugation is promoted by Ulp and SUSP/SENP proteases. Free SUMO can be recycled for another round of protein conjugation (step 7).

hyperactive SUMO-specific proteases known as SUSPs or SENPs [2, 5] (Figure 2 and Table 1).

The first reported molecules covalently modified by SUMO-1 were the GTPase-activating protein 1 (RanGAP1) [6, 7] and the promyelocytic leukemia protein (PML), a main component of nuclear bodies (NBs) [2, 8]. In contrast, SUMO-2 was initially predicted to be a SUMO modifier *in silico*. SUMO-2 was subsequently isolated and its capacity to be conjugated to substrate proteins demonstrated [9, 10]. Interestingly, SUMO-2 and SUMO-3 seem to be involved specifically in the stress response and are able to form chains on target proteins through internal lysine residues, as it is observed with ubiquitin [11]. SUMO-1 has also been found integrated in chains with SUMO-2/-3 but the architecture of these polymers is still unclear [12]. With such large diversity of chains, it should be possible to distinguish between chains types when attached to distinct substrates. The chain recognition by the SUMO-interacting motifs (SIMs) is, therefore, crucial to connect with distinct molecular functions. The knowledge of motifs, recognition signals, and targets regulated by SUMOylation will offer the possibility to integrate individual and global functions controlled by this PTM.

Since the initial demonstration that SUMO was able to modify RanGAP1 and PML, SUMOylation has been involved in multiple cellular processes including the regulation of transcription factor activity, nuclear receptors (NRs), and their coregulators. Proteomic and protein-targeted approaches have revealed a number of SUMOylated corepressors linked to histone deacetylation, demethylation,

Table 1: SUMO/Sentrin specific proteases. SUSPs/SENPs implications and functions. Adapted from Wilkinson and Henley, 2010 [3].

Species	Name	Tissue expression	Localization	Preference	Processing	Deconjugation	Chain editing
S. cerevisiae	Upl1	NA	Nuclear periphery	NA	Yes	Yes	No
	Upl2	NA	Nucleoplasm	NA	No	No	Yes
Mammals	SENP1	Testes (high), pancreas, spleen, liver, ovaries, small intestine, thymus (low).	Nuclear pore and Nucleoplastic speckles	S1 > S2/3	Yes	Yes	No
	SENP2	ND	Nuclear pore	S2/3 > S1	Yes	Yes	No
	SENP3	ND	Nucleolus	S2/3	ND	Yes	No
	SENP5	ND	Nucleolus	S2/3	Yes	Yes	No
	SENP6	ND	Nucleoplasm	S2/3	No	No	Yes
	SENP7	Testes (high), pancreas, ovaries, colon, peripheral blood.	Nucleoplasm	S2/3	No	No	Yes

and other chromatin complexes [13–15]. Implications of SUMOylation in genome integrity, DNA repair, and replication have also been reported [13]. Therefore, it is not surprising to confirm that SUMOylation is implicated in several human disorders such as neurodegenerative diseases associated to huntingtin, ataxin-1, tau, alpha-synuclein, DJ-1 or PARK-7 (Parkinson's disease 7), and superoxide dismutase 1 (SOD-1). SUMOylation has been associated as well with cancer development and tumorigenesis due to its multiple cancer-related targets such as p53, pRB, p63, p73, and Mdm2 [2, 16, 17].

To understand how SUMOylation can specifically control protein activity, it is crucial to explore individual and global processes regulated by this PTM. When studying SUMOylation some of the first questions, we should answer are which technical approaches can be considered?, which biological model and experimental design will be optimal?, and which physiological condition/stimuli can provide conclusive results? The assessment of the advantages and inconveniences of the methods used to explore SUMOylation is crucial to obtain the right answers. Determining which sequences are recognized for the SUMOylation of a target protein and which domains of the "receptor protein" are involved in the recognition of the modified protein is just the first step in this long knowledge acquisition process. When it comes to identify SUMOylated proteins by mass spectrometry (MS), the chosen approach will be critical to distinguish between putative SUMOylated targets from real SUMO substrates that are effectively modified in living cells. In this review, our aim is to provide guidelines for choosing methods to explore protein SUMOylation, to define *cis* and *trans* sequences involved in SUMO-regulated process, and to identify and analyze in an integrated manner, known and putative targets of SUMOylation.

2. Caveats to Study SUMOylation

The presence of active SUMO-specific proteases (SENPs) which remove SUMO from protein substrates, within the cell but also after cell lysis, has been the main problem to study protein SUMOylation (Table 1). Therefore, many of the strategies currently used aim to bypass the action of these proteases. SENPs belong to a family of cysteine proteases with a catalytic triad composed of Cysteine, Histidine, and Aspartic acid residues. The first identified SENP was ULP1 in *S. cerevisiae* [18], and to date six SUMO-specific peptidases have been identified in human cells, namely, SENP 1, 2, 3, 5, 6, and 7 [19, 20]. Recently, a new type of SUMO protease was identified named DeSUMOylating Isopeptidase 1 (DeSI-1) that recognizes a different set of substrates than SENPs [21]. The SUMO proteases are able to cleave the peptide bond to generate the mature form of SUMO, and also an isopeptide bond to deconjugate SUMO from its target proteins. The processing of SUMO to the mature form exposes a C-terminal Gly-Gly motif required for the subsequent activation of SUMO and deconjugation step. Within the cell, some SENPs might be involved in either processing or deconjugating process due to the inherent characteristics of individual enzymes or their differential cellular localization. SUMO proteases are not affected by ubiquitin aldehyde (Inhibitor of De-ubiquitylating enzymes used at 1 μM), or by PMSF (phenylmethanesulfonylfluoride, an inhibitor of serine proteases used at 1 mM) [18, 22]. The most commonly used SENPs inhibitors, NEM (*N*-Ethylmaleimide) and IAA (2-Iodoacetamide), are not specific since they block all cysteine proteases [23, 24]. However, those inhibitors are not cell permeable and need to be used during cell lysis. More recently cell-permeable cysteine protease inhibitors such as the PR619 have been developed [25, 26]. Using the cell permeable protease inhibitor PR619 could result in an accumulation of SUMOylated proteins, some of which can be degraded by proteasome (Rodriguez MS, unpublished observations). SUMOylation was not initially linked to the degradation of target proteins. The first case has been referred for PML upon arsenic trioxide treatment [27, 28]. Uzunova and collaborators reported that the inhibition of proteasome leads to the accumulation of proteins modified by ubiquitin and SMT3 in yeast or SUMO-2/3 in human cells [29]. Therefore, SUMO-2/3 conjugation and the ubiquitin-proteasome system are tightly integrated and act in a cooperative manner. Altogether, these results

show that SUMOylation plays a more important role in protein degradation than previously thought.

One important concept to consider when studying SUMOylation is the inducible nature of this process. While basal level of SUMOylated proteins can be observed in different cell types, it can significantly increase after a proper stimulation. The first evidences that SUMOylation was involved in cellular stress responses was reported by Saitoh and Hinchey [11]. These authors also proposed a distinct regulation for SUMO-2/-3 compared to SUMO-1 and suggested that the SUMO-2/-3 pathway may constitute an element of the cellular response to environmental stress, such as osmotic and oxidative stress and heat shock, to globally increase SUMOylation level [11]. Heat shock was revealed to be very effective for activating SUMOylation by SUMO-2 and SUMO-3 isoforms [11, 30]. Regarding oxidative stress, it was initially reported that high H_2O_2 concentration (100 mM) increased SUMOylation, and on the other hand, low concentrations (<1 mM H_2O_2) inhibits global SUMOylation by inducing the formation of a reversible disulfide bridge between the catalytic cysteine residues of the E1 and E2 enzymes [30]. It has also been described that arsenic (As_2O_3) leads to SUMO-dependent ubiquitin-mediated proteolysis of the PML-RAR fusion protein [27, 28]. This process is mediated by the Ring finger protein 4 (RNF4), a member of the family of SUMO Targeted Ubiquitin Ligases (STUbLs) [31, 32]. RNF4 has the ability to recognize polySUMO chains conjugated to PML and promote its ubiquitin-mediated proteolysis [27, 28].

3. Strategies to Study SUMOylation

SUMO molecules can be associated to proteins through non covalent or covalent interactions [33]. The type of interaction investigated defines the approach to be used and it is crucial to understand the function of SUMO-interacting factors or SUMOylated proteins. The noncovalent interactions with SUMO are mediated by SIMs or by SUMO-binding domains (SBDs), whereas the covalent interactions are mediated by sequences that promote the conjugation of SUMO to target proteins. A combination of deletions and site-directed mutagenesis is a common strategy used to identify these sequences [34–37]. This approach also allows functional SUMOylation studies when the same mutants and deletions are transiently expressed in cell lines and compared to the wild-type proteins [34, 36, 37]. Using one of the SUMO consensus search programs cited here, the lysine residues modified by any of the SUMO proteins can be identified. While the search of putative SUMOylation sites is simple with the help of prediction programs (see below), in many cases, those sites cannot be trusted because programs do not consider several aspects that affect SUMOylation. Among them is, the correct exposition of the consensus sequence, the association with the right partners or the proper location in a cellular compartment. Furthermore, other posttranslational modifications, such as ubiquitylation or phosphorylation, might condition this event [38–40]. Therefore, the combination of multiple approaches is often required to confirm SUMOylation and analyze the functional consequences of this posttranslational modification.

After the identification of the SUMO conjugating enzyme Ubc9 and the SUMO activating enzyme (SAE), one of the most popular techniques used to study SUMOylation was the *in vitro* conjugation assay [41]. This type of assays facilitates the identification of potential candidates of SUMOylation, since in saturating conditions of the substrate, SUMO modifiers (SUMO-1, SUMO-2, or SUMO-3), E1 and E2 enzymes, the SUMO E3 is not required. Nevertheless, if the specific SUMO-E3 is known for the analyzed substrates, its presence increases the efficiency of modification (Figure 2) [42]. The *in vitro* SUMOylation assay is relatively simple to set up and multiple reactions can be performed using several protein substrates and mutants, facilitating the mapping of the modified lysine residues and the analysis of the sequences required for optimal modification. Several commercial sources distribute enzymes and modifiers required to perform *in vitro* SUMOylation assays. The specific substrates can be either generated as recombinant proteins or transcribed/translated *in vitro* using a cDNA encoding the protein of interest. In both cases, the result can be analyzed by PAGE-Western-blot detection using specific antibodies or by labeling the protein of interest with Met^{S35} during the translation procedure. To increase the signal detected, alternative/additional amino acids can be labeled in the protein of interest. The use of radioactive assays provides clean results, and the relative abundance of modified proteins with respect to the unmodified material is preserved. In contrast, Western-blot analysis tends to be more expensive as it implies the use of specific antibodies against analyzed substrates and SUMO-modifiers. Furthermore, detection by Western-blot provides nonlinear saturated signals and blurry images. Finally, if *in vitro* assays are regularly used, the purification of recombinant SUMO modifiers and enzymes is straightforward and affordable.

To clearly demonstrate that a target protein is SUMOylated, in addition to *in vitro* evidences, *in vivo* approaches are essential. Initial studies were based on the detection by Western-blot of specific SUMOylated proteins using antibodies against the protein of interest [6, 8]. First, the protein was immunoprecipitated using specific antibodies, and then analyzed by PAGE-Western blot detection with anti-SUMO antibodies. However, antibodies generally made with nonmodified recombinant protein, in many cases, do not immunoprecipitate the SUMOylated form of a protein. Therefore, if this approach is used, several monoclonal and polyclonal antibodies should be tested. More recently, antibodies recognizing peptides modified by ubiquitin have been developed [43–45], suggesting that this technical alternative should be possible for SUMO-modified peptides. Without any doubt, the most common approach to study SUMOylation has been the nickel chromatography using the different Histidinylated (His6) versions of SUMO molecules. The use of denaturing conditions, with guanidinum and urea in the lysis and washing buffers, results in removal of most unspecific contaminants and inactivation of the SUMO proteases. Preliminary experiments can be set up by transiently expressing His6-SUMO molecules together

with target proteins of interest. However, it will be more convenient to detect SUMO-modified forms from cells stably expressing His6-SUMO [46]. Also, the use of a correct cell environment to analyze SUMOylation can be critical since some events are cell type and/or stimuli specific. It is always convenient to include a positive control such as a typical substrate of SUMOylation (e.g., PML, RanGAP1, IκBα, or p53). To increase the level of SUMOylated proteins, a relevant stimulation can be considered, as well as pretreatments with proteasome inhibitors. More recently, the use of SUMO-interacting motifs (SIMs) from the RNF4 SUMO-dependent ubiquitin ligase has been developed to capture SUMOylated proteins. This approach looks very promising to capture SUMOylated proteins and also SUMO-interacting cellular factors due to the nondenaturing conditions used. However, it remains to be investigated if the nature of the SUMO-chains captured by these SIMs is limited to the particular SUMO-chain architecture recognized by RNF4. The putative SUMOylated proteins purified following these approaches are subsequently analyzed by Western-blot or by MS to identify the isolated SUMO-conjugated cellular factors.

In order to visualize the sites of SUMO conjugation, an "*in situ* SUMOylation assay" was developed [47]. This assay consists in five steps: (1) culture of mammalian cells on a coverslip; (2) permeabilization of the cells with detergents; (3) incubation for SUMOylation reaction using GFP/YFP-tagged SUMO, E1 and E2 (Ubc9) enzymes, and ATP; (4) washing out of soluble materials including unconjugated GFP/YFP-SUMO; (5) fixation of the cells to stop the reaction. Muramatsu et al. recently simplified this technique, by using, instead of recombinant proteins, only cultured cells and crude bacterial lysate containing GFP-SUMO-1 [48]. Using the *in situ* SUMOylation assay, it was found that both nuclear rim and PML bodies, besides mitotic apparatuses, are major targets for active SUMOylation. The ability to analyze possible SUMO conjugation sites should constitute a valuable tool to investigate where SUMO E3-like activities and/or SUMO substrates exist in the cell. Moreover, the simplified form of this assay could be useful in large-scale screening approaches for the identification of drugs that can inhibit or enhance SUMOylation.

Fluorescence resonance energy transfer (FRET) is a process by which the excited state energy of a fluorescent donor molecule is transferred to an acceptor molecule. Efficient energy transfer requires very close proximity and can, therefore, be used as a read-out for covalent and noncovalent protein interactions. FRET experiments have effectively detected the association of ubiquitin [49] or SUMO [50, 51] with their target proteins. However, the full potential of FRET methods is often limited due to photobleaching, autofluorescence, and high residual excitation of the acceptor fluorophore. This assay has applications in SUMO protease characterization, enzyme kinetic analysis, determination of SUMO protease activity in eukaryotic cell extracts, and high-throughput inhibitor screening [52, 53]. Ran-GAP1 tagged to Cyan fluorescent protein (CFP) and yellow-fluorescent-protein- (YFP-) tagged mature SUMO were used in the first assays. RanGAP1 was chosen because it is one of the most efficient SUMO targets not requiring addition of an E3 ligase [7]. FRET assay was also used to measure the interaction between SUMO-1 and C/EBPβ in primary astrocytes and evaluate how SUMOylation of C/EBPβ can regulate NOS2 expression in neurological conditions and diseases [54]. The role of SUMO modification on the localization and the activity of the orphan nuclear receptor LRH-1 (liver receptor homologue 1) was also studied using FRET [55]. In 2011, the group of Liao reports the HTS assay development in living cells using an engineered FRET pair, CyPet and YPet, to determine the K_d of SUMO-1 and Ubc9 interaction, which fits very well with that determined by other methods, such as surface plasmon resonance (SPR) [56]. The same FRET pair, CyPet, and YPet, has been used to develop a pioneer cell-based technique in the field, FRET HTS. Both K_d determination and cell-based HTS were performed in 384-well plate format, which readily allows repeated study and large-scale application, such as genome-wide and industrial applications. Invitrogen Discovery Assays and Services reported recently the development and application of time-resolved Fluorescence-resonance-energy-transfer- (TR-FRET-) based assays capable of detecting SUMOylation or deSUMOylation in a high-throughput screening (HTS) format. Protein SUMOylation can be detected using LanthaScreen (Invitrogen, Carlsbad, CA) TR-FRET technology. Additionally, they have generated reagents useful for assessing the deSUMOylation activity of a SUMO-specific protease [57].

Bioluminescence resonance energy transfer (BRET) methods have been developed to overcome some limitations of FRET [58, 59]. Compared to FRET, which often uses two fluorescent proteins, BRET methods do not require external excitation and, therefore, have relatively low background signal intensities, allowing for more sensitive detection of energy transfer during experiments. An *in vitro* BRET-based detection system of SUMOylation was developed using RanGAP1 as SUMO substrate. Components of the BRET system include Renilla luciferase (Rluc) fused to SUMO, as the energy donor and enhanced yellow fluorescence protein (EYFP) fused to RanGAP1, as the energy acceptor. BRET efficiencies were determined in the presence of E1 (SAE1/2) and E2 (Ubc9) enzymes. The efficiency of this assay was confirmed by gel electrophoresis and compared with FRET system under identical conditions [60]. Without requiring any external photoexcitation, BRET system showed 3-fold higher RET efficiency than an almost identical FRET system.

Proximity Ligation Assay (PLA) is a method allowing specific imaging of individual protein or protein complexes in tissue samples [61]. This method depends on two recognition events. First, the formation of a proper detection complex that results in the creation of a circular DNA strand, which is used to template a localized RCA (rolling-circle amplification) reaction. This will generate a long single-stranded DNA molecule, rolled-up in a ball that can be detected by hybridizing fluorescence-labeled probes. The binding to a target molecule or complex by two antibodies with attached oligonucleotides, referred to as proximity probes, is followed after washes by the addition of two more oligonucleotides that are then ligated into a circular DNA strand, templated by the oligonucleotides

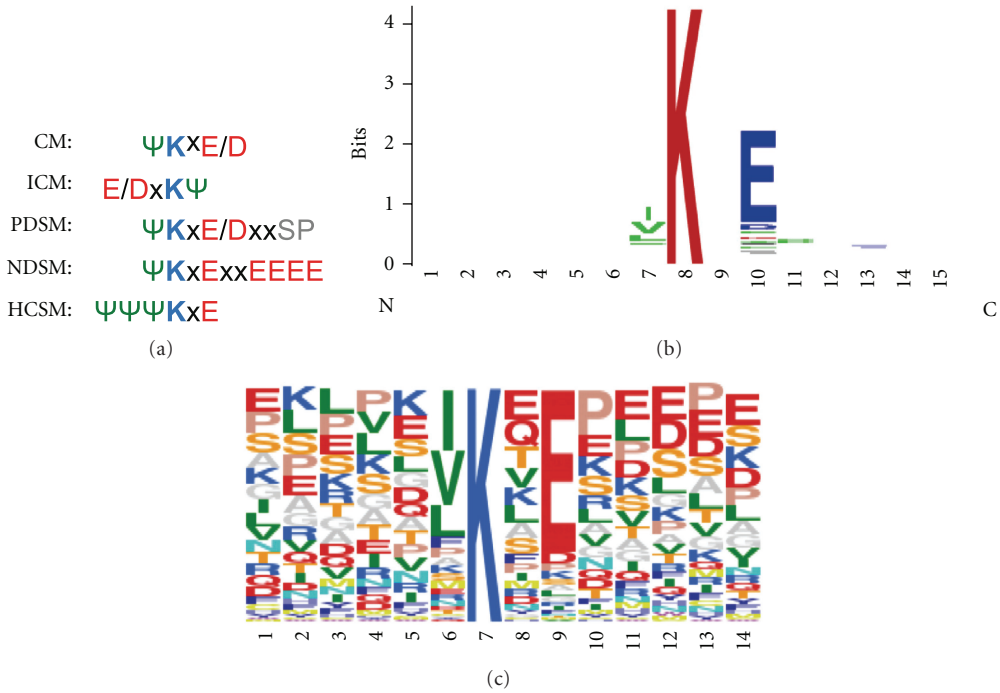

Figure 3: Sequence alignment SUMO consensus motifs. (a) Amino acid sequence alignment of the canonical SUMO consensus motif (Ψ represents a hydrophobic amino acid, K is the Lys modified by SUMO and x represents any amino acid). CM: canonical consensus motif. ICM: inverted consensus motif. PDSM: phosphorylation-dependent SUMO motif, NDSM: negatively charged amino-acid-dependent SUMO motif, HCSM: hydrophobic cluster SUMO motif. Amino acids in blue: basic, red: acid, green: hydrophobic, gray: phospho serine. (b) WebLogo [64] representation of the consensus motif of SUMOylated proteins reported in the phosphosite database on Fri Feb 03 08:31:18 EST 2012 (PhosphoSitePlus [65], http://www.phosphosite.org/). (c) The same SUMO motif aligned using Sequence Logo. Amino acid sequences are represented by frequency on the identified consensus.

attached to antibodies. Next, one of the antibody-bound oligonucleotides is used to prime an RCA reaction, resulting in the formation of a single-stranded rolling circle product (RCP). The RCP is composed of concatenated complements of the DNA circle, and it is covalently attached to one of the proximity probes. The RCP is then visualized by hybridization of fluorescence-labeled complementary oligonucleotide detection probes. In *in situ* PLA, pairs of antibodies are required to ensure higher selective detection and allowed the formation of a brightly fluorescent spot, which can be imaged by microscopy. In a similar manner, the requirement for two proximal recognition reactions by antibodies can also be used to investigate interactions among pairs of proteins, each of which is recognized by one antibody, or secondary modifications like phosphorylations or glycosylations, by using the appropriate affinity reagents. *In situ* PLA requires proximity between epitopes in order to allow formation of an amplifiable circulized ligation product and is suitable for any protein pairs for which antibodies are available. PLA offers at least two advantages over FRET or BRET experiments, first endogenous proteins can be investigated and second, signal amplification by RCA increases the number of fluophores per detected protein interaction, so that single events can be easily visualized as prominent fluorescent spot while ignoring any nonspecifically bound fluorescent probes [61, 62]. Recently, PLA was adapted to localize SUMOylated protein. In this assay, primary antibodies directed against GFP and SUMO-2/-3 and secondary antibodies labeled with oligonucleotides were employed to reveal the location of SUMOylated ZBTB1 [39]. Altogether, this method should contribute to the establishment and use of comprehensive interactome maps in basic research and for clinical diagnosis.

4. Sequences Recognized by the SUMOylation System

Early studies allowed the identification of a potential sequence for protein SUMOylation with the first reported SUMO-modifier, SUMO-1 [8, 40]. The sequence ΨxKE/D considered as SUMO consensus motif (CM), where Ψ is a hydrophobic amino acid, x any amino acid, K a lysine and E/D a glutamic or aspartic amino acid, favored identification of multiple substrates (Figure 3). The development of bioinformatic tools contributed to increase the long list of substrates of SUMO-1, SUMO-2 and SUMO-3. Among the most popular programs are SUMOplot (http://www.abgent.com/tools/sumoplot/) and SUMOsp (http://sumosp.biocuckoo.org/). However, predicted SUMOylation sites using these tools have not always been confirmed. As mentioned above, other structural, temporal, or cellular distribution requirements are important and not considered by these software tools. With the use of new approaches, and in particular with the contribution of

MS, the SUMO modification motif was recently corrected [39]. Nowadays, we know the existence of an inverted consensus motif (ICM), a phosphorylation-dependent SUMO motif (PDSM), where the phosphorylated serine is located at 5 amino acids distance from the modified lysine, a negatively charged amino acid-dependent SUMO motif (NDSM) and a hydrophobic cluster SUMOylation motif (HCSM) that increases the efficiency of modification in relevant targets of SUMOylation such as RanGAP1 [38, 39] (Figure 3). Here, we have analyzed all SUMO motifs present in the SUMOylated human proteins that have been reported in the PhosphoSitePlus [63] (http://www.phosphosite.org/) and found that the most frequent SUMO consensus contains the sequence [IVL]KxE (Figure 3).

It is important to underline that only a small proportion of these proteins have been confirmed by mass spectrometry through identification of the SUMO-GG signature peptides. Therefore, it is crucial to distinguish between potential SUMOylated substrates identified using *in vitro* assays and overexpression systems from those sites identified *in vivo* with an unambiguous mass accuracy (see the following section). SUMO can also interact with proteins in a noncovalent manner due to the presence of SIMs. The first evidence of SIMs was published by Minty and collaborators in 2000 [35]. Using a two-hybrid approach, the authors observed that some proteins were able to interact with the SUMOylated version of p73, a member of the p53 family. This analysis revealed a common SxS sequence, in which x is any amino acid surrounded by two serine residues, flanked by a hydrophobic core on one side and acidic amino acids on the other. A few years later, it was found that the presence of a Val/Ile-x-Val/Ile-Val/Ile (V/I-x-V/I-V/I) motif could allow the interaction of SUMO with SIMs [36]. Several proteins, like the SUMO ligases PIASX and Ran binding-protein 2 (RanBP2/Nup358), contain this motif [36]. SIMs are also found in some SUMO substrates raising the possibility that components of the modification pathway interact noncovalently with SUMO to facilitate its transfer from enzymes to substrates. In support of this, the SIM in RanBP2/Nup358 is directly adjacent to the minimal IR1-IR2 domain that has E3 activity. However, although this SIM has been shown to bind SUMO, it does not appear to be essential for E3 activity *in vitro* [66]. The hydrophobic core of a SIM can bind to an interaction surface on SUMO via a parallel or antiparallel orientation. The acidic residues adjacent to the core might contribute to the affinity, the orientation or the paralogue specificity of binding [67, 68]. From these initial reports, a more complex type of SIMs named SUMO-binding domains (SBDs), containing several hydrophobic cores of 3 to 4 residues often surrounded by a cluster of acidic amino acids was born [37, 69]. Recent analysis performed by Hoffman revealed 3 different types of SIMs with the following PROSITE format: SIMa) (PILVM)-(ILVM)-x-(ILVM)-(DES>) (3), SIMb) (PILVM)-(ILVM)-D-L-T, and SIMr) (DSE) (3)-(ILVM)-x-(ILVMF) (2) [70]. The identification and validation of these SIMs using site directed mutagenesis has been an important approach to investigate the role of SUMO in the regulation of the activity of one particular process or pathway.

5. Analysis of SUMOylated Human Proteins

Multiple strategies have been exploited to purify SUMOylated proteins from human cell lines such as the use of tagged versions of SUMO and the use of a SIM-based capturing system [71]. In contrast to ubiquitin, antibodies against SUMO have not been deeply explored, perhaps due to the poor capacity of the first reported antibodies to immunoprecipate SUMO-modified proteins. Alternatively, HA, FLAG, and Myc tagged versions of SUMO have been used to immunopurify SUMO conjugates. The particularity of the immunoprecipitation and SIM-based capturing system is that both methodologies offer the advantage of isolating SUMO-interacting proteins that could be used to connect with the SUMO-regulated functions. However, in both cases one has to distinguish between SUMO-modified proteins and SUMO-interacting factors. Tagged forms such as His6-SUMO molecules are, therefore, more popular to unambiguously identify sites of SUMOylation and formation of SUMO-polymers. A main advantage is the highly denaturing conditions that can be used with this approach allowing inactivation of SUMO-specific proteases and removal of copurified interacting factors. Nevertheless the nickel beads used in this method also purify endogenous proteins that naturally contain histidine rich sequences. To reduce contaminant proteins, tags in tandem allow more than one purification step, increasing the purity of the fractions. The classical Tandem Affinity Purification (TAP) strategy includes a protein A domain and a calmodulin binding domain separated by a tobacco etch virus (TEV) cleavage site. However, large tags might affect the dynamics of conjugation and deconjugation. To avoid these problems, smaller tags such as biotinylated tags have also been used to purify bio-ubiquitin adducts using avidin or streptavidin resins under denaturing conditions [72, 73]. However, the bio-SUMO counterpart is still under development in drosophila (Mayor Ugo, personal communication). The risk of copurifying endogenous biotinylated proteins cannot be excluded.

Therefore there is no perfect method for purification of SUMOylated proteins and more than one of these approaches should be considered to collect complementary information. For instance, while transient expression experiments quickly reveal potential SUMOylated substrates, the overexpression of ubiquitin-like modifiers favors compensatory mechanisms likely affecting chain architecture [74]. The use of cell lines that stably express tagged molecules represent a better option to approach SUMOylation [46]. Several human cell lines have been used to identify SUMO substrates by mass spectrometry but one has to go through the difficult comparative analysis of published work to verify if a particular protein of interest is a putative target of SUMOylation. Apart from PhosphoSitePlus, data base that regularly updates SUMOylated proteins that have been found using multiple strategies, there is not a single database that includes all putative SUMOylated proteins identified by mass spectrometry. This is perhaps due to the fact that while the identification of a protein by mass spectrometry is unambiguous, there is no SUMO acceptor lysine identified by mass

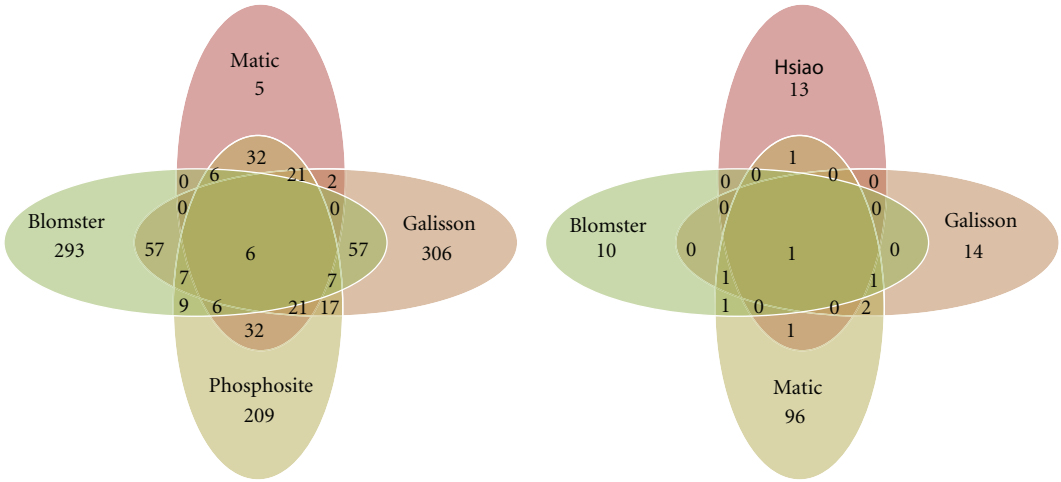

Figure 4: Comparative analysis of SUMO-modified proteins. (a) All proteins reported to be SUMOylated in the literature and at PhosphoSitePlus database (http://www.phosphosite.org/) were manually extracted and compared to those found by MS in 3 recent studies [39, 75, 76]. The protein list in the PhosphoSitePlus includes proteins for which the site of SUMO modification was not determined by MS. All protein names and accession numbers were first mapped to Uniprot accession numbers by using mapping data downloaded from ENSEMBL. Next, all Uniprot accession numbers were mapped to HGNC symbols and HGNC symbols for each study were uploaded to MySQL database. This means that all protein accessions that mapped to the same HGNC symbol were considered as redundant for the comparative analysis provided here. Finally, the necessary MySQL queries were made to define overlapping HGNC symbols between the different resources and the output used for creating the presented SUMO protein Venn diagram. List of proteins identified by other authors and confirmed by Matic et al.: PSMD12, TRIM24, CD3EAP, SART1, MYO1B, BRD4, SF3B1, LMNA, HNRNPC, PARP1, TOP1, KRT5, FOSL2, FLNA, MAP4, CANX, PML, STAT1, MKI67, RANGAP1, YLPM1, RBM25, RANBP2, VASP, HNRNPM, ADAR, ACTB, SUMO2, SUMO1, GTF2I, KHDRBS1, RLF, TRIM28, TCOF1, NAB1, SAFB2, NUMA1, IFI16, ZNF800, ARID4B, ZMYM1, ZMYM4, PTRF, PBRM1, CCAR1, RBM12B, FNBP4, ZBTB38, ZNF280C, KDM2B, GEMIN5, RREB1, SYMPK, ZBTB9, THOC1, ERBB2IP, RSF1, HNRNPUL1, PNN, BCLAF1, ACIN1, ZNF295, ZMYND8, TRIM33, ZBTB1, ZNF451, ACTG1, ACTB. Proteins considered in this analysis are included in the Supplementary Table 1. (b) Comparative analysis of SUMOylation sites. All peptide sequence reported with annotated SUMOylation sites based on mass spectrometry data from Matic et al. [39], Galisson et al. [76], Hsiao et al. [77], and Blomster et al. [78] were manually extracted. For each SUMO-modified site, six flanking amino acid residues on both sides were extracted. The resulting 13 amino acid residue sequences from each of the above mentioned studies were uploaded to an MySQL database and the necessary queries for comparing the peptides between studies were performed and used as input for the creation of the SUMO peptide Venn diagram.

spectrometry for most SUMO target proteins reported. Furthermore, including in a single list, proteins that have been found in different cell lines under a different stimulation condition perhaps do not make much sense. Nevertheless, we have compared 3 recent studies that use His6-SUMO-2/MS approach to the list of SUMOylated proteins included in the PhosphoSitePlus [39, 75, 76]. The work reported by Matic et al. is significant as it represents the largest collection of peptides containing the SUMOylation signatures. The number of overlapping proteins between these 3 sets is low (only 6 out of 300 proteins analyzed, corresponding to more than 600 modification sites) integrated on PhosphoSitePlus [65], a large proportion of the SUMOylated proteins have not been confirmed by mass spectrometry (Figure 4(a)). The list of proteins considered in this analysis and overlapping data sets are included in the Supplementary Tables 1 and 2 (available online at doi:10.1155/2012/875148).

The recent use of quantitative proteomic approaches has significantly improved the quality of the data sets and our knowledge on the SUMO-induced processes [79]. The stable isotope labeling by amino acids in cell culture (SILAC) employs stable isotopic variants of amino acids for metabolic labeling of endogenous proteins and subsequent quantification [80, 81]. Control and treated cell lines are differentially labeled using isotopic variants of arginine and lysine. Cell lysis of control and treated cells mixed in normally 1 : 1 ratio is performed under denaturing conditions to inactivate proteases and reduce the number of contaminant proteins. The trypsin digestion precedes the analysis of the digested peptides by mass spectrometry. Protein identification is performed by searching (MS/MS) spectra against protein databases. Quantitation is obtained by extracting the intensity from survey scans of the unlabelled and stable isotope labeled version of each identified peptide. Absolute quantification (AQUA) employs labeled marker peptides that are spiked at known concentrations to enable absolute quantifications [82, 83]. Labeling can also be performed after cell lysis using chemical methods such as isobaric tags for relative and absolute quantification (iTRAQ) [84]. In all cases, control cell populations are considered in the experimental design to distinguish between target proteins and contaminants. Despite the efforts of the international community, the number of SUMOylation peptide signatures remains low. In contrast to the ubiquitylation GG signature,

the SUMOylation signature is larger, complicating the identification of these peptides. Several strategies have been used to overcome this problem, but the most successful one introduces artificial trypsin cleavage sites to generate short SUMO-derived peptides [39]. A comparison of four studies where SUMOylation signature peptides have been reported is illustrated in Figure 4(b) and Supplementary Table 3. Two main observations can be underlined: less than 150 sites have been identified in total and little overlap exists between the identified SUMOylation sites. The limited overlap can be due to the fact that different cell lines, treatments and strategies have been used in those studies, reducing the chances to isolate similar peptides. A big effort has to be done to improve the identification of SUMOylation signatures. In the ubiquitin field the use of antibodies against the GG-signature have significantly improved the databases of ubiquitin-GG signatures [43–45]. Perhaps the development of antibodies that could recognize SUMOylation signature motifs might be helpful for the identification of SUMO acceptor lysines.

6. Integration of SUMO-Regulated Processes

The analysis of SUMO conjugates *in vitro* and *in vivo* has extensively been used in the field to demonstrate the SUMOylation of target proteins. Such information, included in the PhosphoSitePlus [65], has been integrated here together with the one obtained in three mass spectrometry (MS) studies [43–45] (Supplementary Table 1) using the Ingenuity Pathway Analysis software (IPA) (http://www.ingenuity.com, Ingenuity Systems, Redwood City, CA, USA). IPA integrates putative and proven SUMO substrates into several pathways [85] such as Ran-signalling (Figure 5), p53 (Figure 6), Ubiquitin-signalling (Supplementary Figure 1), and Glucocorticoid signalling pathways (Supplementary Figure 2). The main diseases and disorders associated to the integrated proteins are in a decreasing order: cancer, reproductive system disease, infectious diseases, genetic disorders, and respiratory diseases. The top molecular functions related to this set of proteins are indicated in Figure 7(a) and Supplementary Table 4 and include Gene Expression, cell death, cell cycle, and DNA replication, recombination, and repair, among others. More interesting, among the top canonical pathways indicated in the Figure 7(b) and Supplementary Table 5, several links to transcription regulators such as MYC, E2F1, TP53, RB1, and hypoxia-inducible factors can be found. The positive or negative impact of SUMO in transcription has been largely documented. SUMOylation was shown to have an impact on transcription regulators (e.g., IκBα) [40] or directly on transcription factors (e.g., p53) [86]. However, a large majority of studies has identified a functional role of SUMOylation in transcriptional repression [14]. It is known that SUMOylation can regulate transcription at multiple levels, including DNA binding, subcellular localization, interaction with coregulators and chromatin structure. SUMOylation of transcription repressors and corepressors, seems to be quite a general mechanism to recruit chromatin remodeling and histone-modifying complexes involved in repression [87]. A number of chromatin modifying complexes exhibit a combination of SUMO conjugation sites with SIMs in the same or different subunits, we can envisage a role of SUMOylation in the assembly or the stability of these complexes [88]. In addition, SUMOylation of transcription factors creates new interaction surfaces for chromatin-modifying machineries that eventually may convert activators into repressors, as it has been indicated for p300 or Sp3 [88].

Several cellular factors of the same signaling cascades have been identified within the analyzed lists of proteins supporting the role of SUMO in the regulation of these pathways. In the Ran pathway (Figure 5), p53 (Figure 6), Glucocorticoid Receptor (Supplementary Figure 1), and Ubiquitin-Proteasome pathway (Supplementary Figure 2), proteins that have been identified as putative SUMO targets (in gray) from those that have not (in white) are clearly predominant or abundant. These findings suggest that typical activators of these pathways might have an impact on the SUMOylation of these putative or proven substrates of SUMO conjugation. SUMOylation can indeed be regulated through multiple mechanisms [89–93]. It has been shown that the expression of various components of the SUMOylation system is regulated under certain physiological or pathogenic conditions. Deyrieux and collaborators [94] have demonstrated that, during keratinocyte differentiation, the SUMOylation system was transiently up regulated by Ca^{2+} signalling. Ca^{2+} induced the transcriptional activation of the genes encoding several components of the SUMOylation system, including SAE1/SAE2, Ubc9, SUMO2/3, and PIASx. Also, it was described that hypoxia can induce the expression of SUMO-1 [95]. The regulation of the expression levels of the components of the SUMO conjugation system and their intrinsic activity can also be modulated by cellular stimuli. Recently, a protein named RSUME (RWD-containing SUMOylation enhancer) has been reported to enhance overall SUMO-1, -2, and -3 conjugations [96]. This protein binds to the E2 enzyme Ubc9 and increases the noncovalent association of Ubc9 with SUMO. This leads to the enhanced Ubc9-SUMO thioester formation and SUMO conjugation. Interestingly, during hypoxia, RSUME expression is induced, leading to an increase of HIF-1α SUMOylation, stabilization, and transcriptional activity. However, a recent study indicates that the hypoxia-induced HIF-1α SUMOylation targets this protein for degradation through the von Hippel-Lindau (VHL) protein-mediated ubiquitin proteasome pathway [97, 98]. The activation of signaling cascades also favors the crosstalk between SUMO and other PTMs. Phosphorylation regulates SUMO conjugation of multiple transcription factors through the PDSM motif [38] (Figure 3), including heat-shock factors (HSFs), myocyte enhancer factor 2 (MEF2), and oestrogen-related receptors (ERRs) α and γ [99–102]. This phosphorylation-dependent regulation of SUMOylation has been referred as a phospho-sumoyl switch [103]. Furthermore, lysine residues involved in SUMOylation are also targets of other PTMs, including ubiquitylation, acetylation, and methylation. For instance, SUMO conjugation can occur on the same lysine residue used to promote ubiquitylation of IκBα resulting in a competition between these PTM [40]. However,

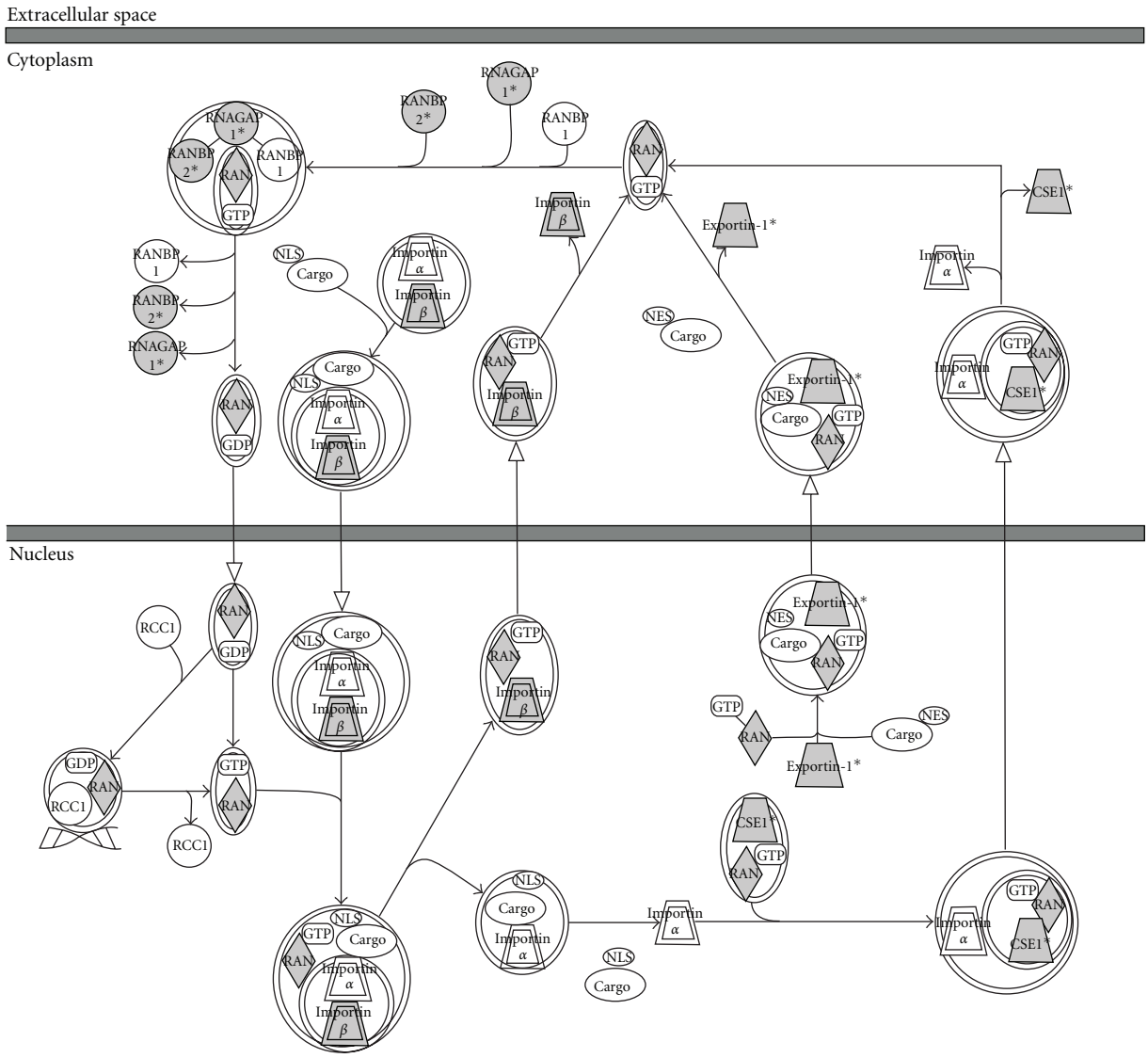

Figure 5: Integrated view of the role of SUMO in the Ran Signalling pathway. Ingenuity analysis of proteins that have been identified (in gray) in recent studies: KPNB1, CSE1L, TNPO1, RANBP2, RAN, XPO1, and RANGAP1 (Figure 4 and Supplementary Table 1) by mass spectrometry using His-6-SUMO-tagged.

SUMOylation and ubiquitylation do not necessarily compete with each other as, in some cases, SUMOylation acts as a recognition signal for an ubiquitin ligase [97]. The interplay between SUMOylation and acetylation has been observed in the regulation of proteins such as MEF2, histone, and hyper methylated in cancer 1 (HIC1) [104–107]. In the case of MEF2, the SUMOylation-acetylation switch is regulated by phosphorylation [105]. Altogether, these data demonstrate that multiple signaling cascades are regulated by SUMOylation with an intensive crosstalk between PTMs.

The type of analysis developed here can be used to visualize individual and global processes regulated by SUMOylation. In this way, the study of SUMO-targets will not be isolated but integrated with the rest of the SUMO-regulated processes. Beyond the identification of molecular processes and signaling cascades, IPA can also be used for the identification of biomarkers of a given process or pathology where SUMOylation plays a critical role (Supplementary Table 6). In the future, this information could help us to identify pathologies, treat diseases, and predict responses to avoid treatments that will activate unwanted side effects. The number of available drugs that potentially affect SUMO regulated processes is not negligible so one can envisage the possibility to use them to tackle signaling cascades, molecular events and/or diseases where SUMOylation is critical (Supplementary Table 6). This approach could accelerate our

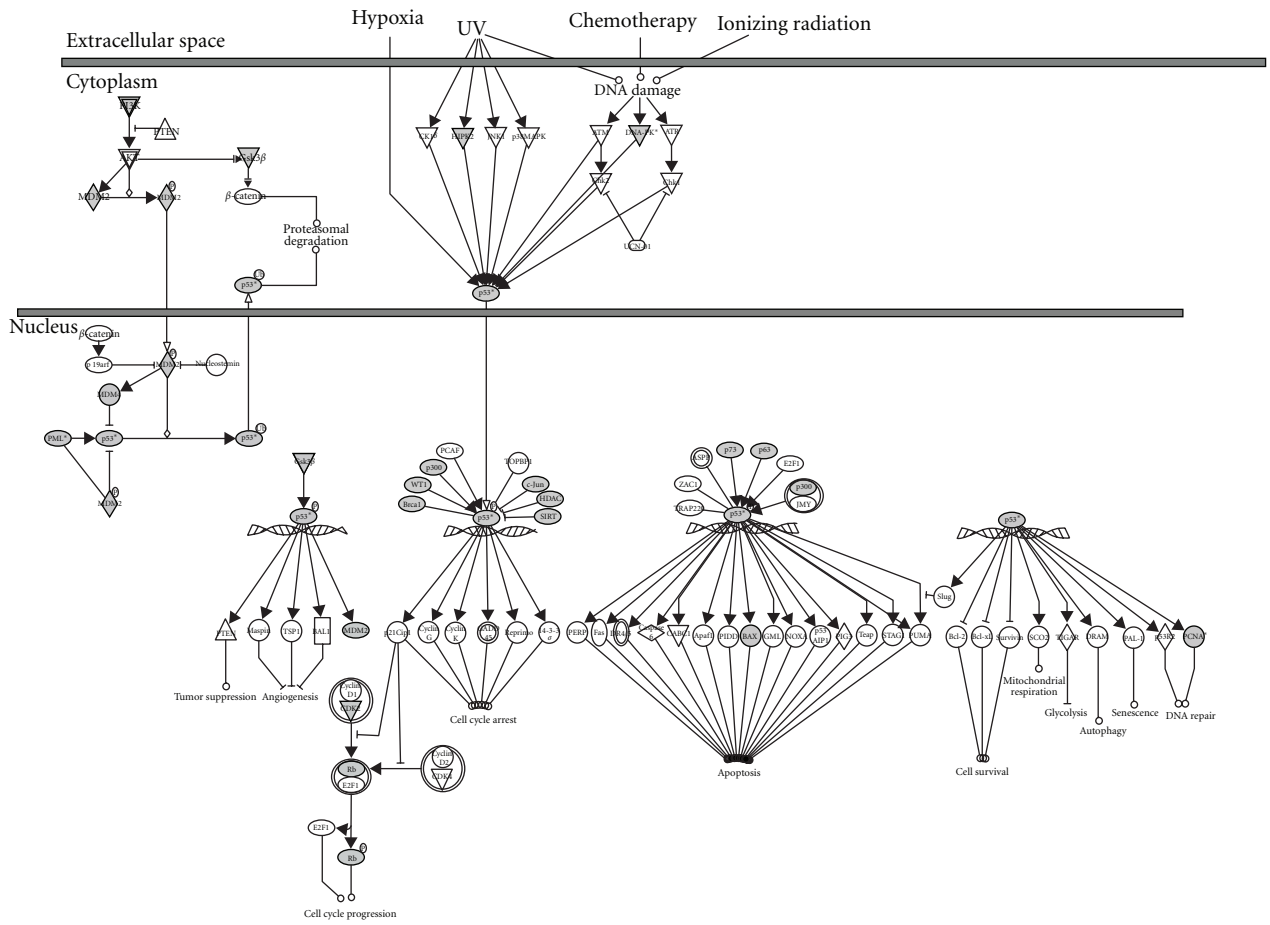

Figure 6: Integrated view of the role of SUMO in the p53 Signalling pathway. Ingenuity analysis of proteins that have been identified (in gray) in recent studies: TP53, WT1, PRKDC, TP63, PIK3C2A, TP73, HDAC1, MDM2, BAX, EP300, RB1, PCNA, MDM4, JUN, GSK3B, HIPK2, PML, BRCA1, CDK2, and SIRT1 (Figure 4 and Supplementary Table 1) by mass spectrometry using His-6-SUMO-tagged.

understanding of the role of SUMOylation in many essential cellular events.

7. Concluding Remarks

SUMOylation just as other PTMs contributes to the regulation of multiple processes in the cell. To investigate the role of SUMO on the function of a given protein or pathway, the main approach considers the identification of the sites of modification or the sequences interacting with SUMOylated proteins. In contrast to ubiquitylation, SUMOylation sites can be predicted using one of the available algorithms published by several groups. However, those programs are not 100% reliable as they do not consider several aspects that regulate the SUMOylation of a protein. Here, we have analyzed all motifs present in human proteins reported in the PhosphoSitePlus (http://www.phosphosite.org/) that have been proven as SUMOylated using multiple approaches and found that most of the proteins contain the consensus [IVL]KxE. Before going through the identification of one substrate or pathway of interest, it is important to verify the public information available. There is not a single database that includes all published information of putative SUMO modified proteins identified by MS. However, the PhosphoSitePlus database includes SUMO sites that have been demonstrated by several groups using several methodologies. It is important to underline that while the lists of proteins identified using MS and other approaches can be counted by hundreds, the number of SUMOylation signatures identified from endogenous modified proteins remain low (no more than 150). All this information can be integrated in a rational manner to identify within a pathway, proteins that have been linked to SUMOylation. More importantly, this type of analysis can be used to identify biomarkers for a given process or disease and/or choose possible targets for therapeutic intervention (Supplementary Table 6). A long list of those targets has been used to develop drugs that can potentially be exploited to characterize processes or pathologies were protein regulation by SUMOylation is essential.

Authors' Contribution

E. D. S. Ferrada and F. L. Otsoa contributed equally to this paper.

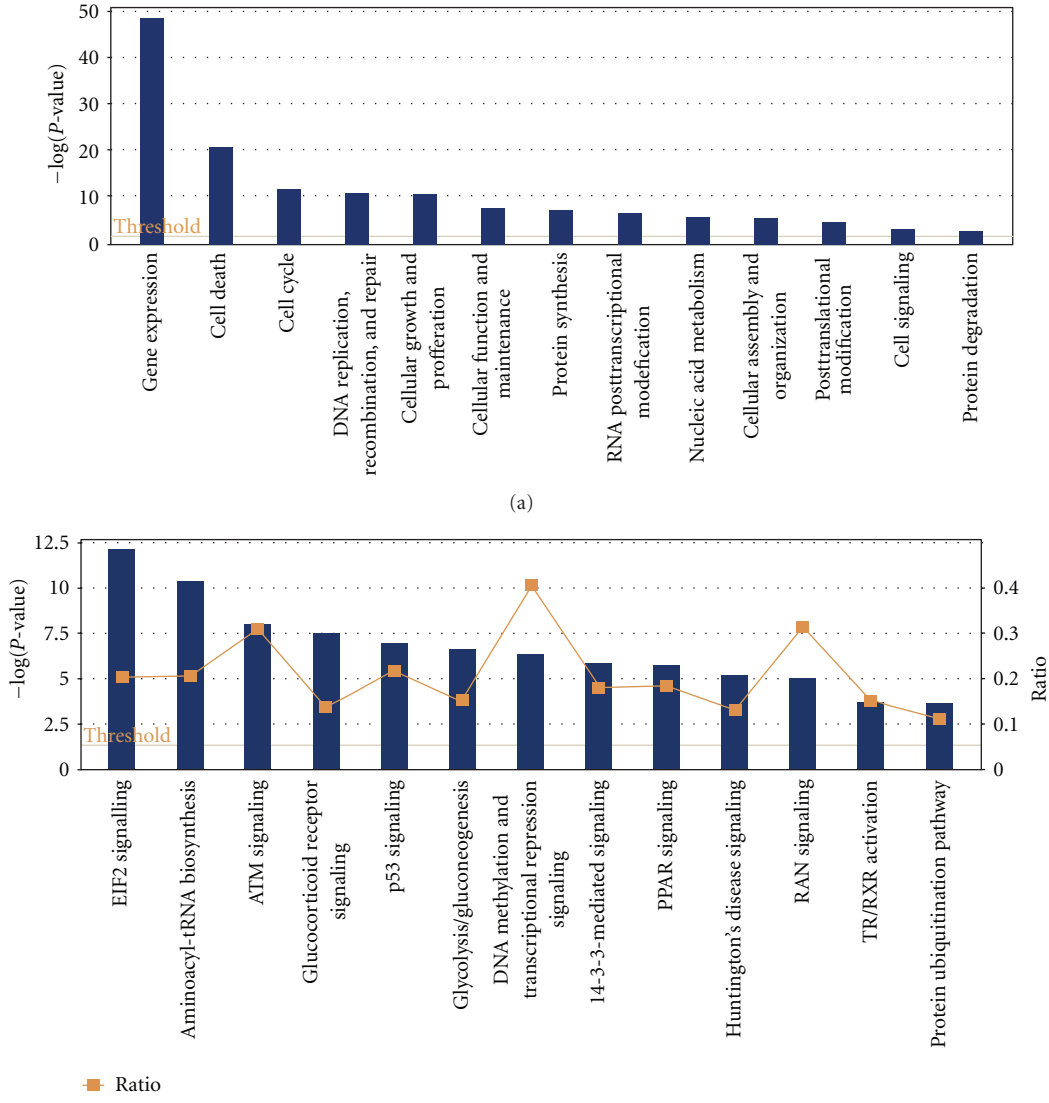

Figure 7: Molecular functions and canonical pathways regulated by SUMOylation. Ingenuity (IPA) analysis of proteins reported to be SUMO-modified in the PhosphoSitePlus (http://www.phosphosite.org/) and 3 recent MS studies [39, 75, 76]. (a) The top molecular functions are indicated. A dominant link to gene expression has been found. All functions are superior to the threshold (yellow line). (b) The top canonical pathways are indicated. All shown pathways are superior to the threshold. The Canonical Pathways that are involved in this analysis are displayed along the x-axis. The right y-axis displays the ratio up to 0.6. The ratio is calculated as follows: number of genes in a given pathway that meet cut-off criteria, divided by total number of genes that make up that pathway. Therefore y-axis displays the results importance. For the ratio, taller bars have more genes associated with the Canonical Pathway than shorter bars. The graph displaying the various pathways is presented from largest ratio to smallest ratio.

Acknowledgments

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Review Article

Ubiquitin C-Terminal Hydrolase L1 in Tumorigenesis

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Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1, aka PGP9.5) is an abundant, neuronal deubiquitinating enzyme that has also been suggested to possess E3 ubiquitin-protein ligase activity and/or stabilize ubiquitin monomers *in vivo*. Recent evidence implicates dysregulation of UCH-L1 in the pathogenesis and progression of human cancers. Although typically only expressed in neurons, high levels of UCH-L1 have been found in many nonneuronal tumors, including breast, colorectal, and pancreatic carcinomas. UCH-L1 has also been implicated in the regulation of metastasis and cell growth during the progression of nonsmall cell lung carcinoma, colorectal cancer, and lymphoma. Together these studies suggest UCH-L1 has a potent oncogenic role and drives tumor development. Conversely, others have observed promoter methylation-mediated silencing of UCH-L1 in certain tumor subtypes, suggesting a potential tumor suppressor role for UCH-L1. In this paper, we provide an overview of the evidence supporting the involvement of UCH-L1 in tumor development and discuss the potential mechanisms of action of UCH-L1 in oncogenesis.

1. Introduction

Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1, aka PGP9.5) is an abundant neuronal protein consisting of 223 amino acids [1]. The best understood function of UCH-L1 is its deubiquitinating enzyme (DUB) activity that catalyzes hydrolysis of C-terminal esters and amides of ubiquitin (Ub) to generate monomeric Ub [2, 3]. In addition to its DUB activity, UCH-L1 has also been suggested to possess a putative, dimerization-dependent E3 ubiquitin-protein ligase activity and/or have a role in stabilizing Ub monomers *in vivo* [4, 5]. As a DUB, UCH-L1 facilitates Ub recycling and, therefore, can regulate the cellular pool of available Ub [6], giving UCH-L1 the capacity to modulate many ubiquitin-dependent cellular processes. Although its exact physiological function remains unclear, a growing body of evidence implicates UCH-L1 in the progression of human malignancies. Currently, the specific role of UCH-L1 in cancer pathogenesis is not known. UCH-L1 has been reported to be upregulated in several tumor tissues and cancer cell lines [7–13] and has been suggested to function as an oncogene in the progression of many cancers including lymphoma [11], colorectal cancer [14], and nonsmall cell lung carcinoma [8]. Conversely, studies have been put forth designating UCH-L1 as a tumor suppressor in the pathogenesis of nasopharyngeal [15] and breast [16] cancer. Despite the controversy regarding the exact function of UCH-L1 in oncogenesis, these studies suggest that UCH-L1 is an important regulator of tumor formation and maturation. Here, we review the current knowledge of the function and mechanisms of actions of UCH-L1 in tumorigenesis.

2. Functions of UCH-L1 in the UPS

The ubiquitin-proteasome system (UPS) is a major intracellular proteolytic pathway that facilitates the degradation of normal cellular proteins as well as the clearance of misfolded and damaged proteins [17]. In the UPS, protein substrates are tagged with polymers of a 76-amino-acid polypeptide, ubiquitin (Ub), followed by recognition and degradation by the 26S proteasome. This process is facilitated by the sequential actions of at least three classes of enzymes: ubiquitin-activating enzymes (E1), ubiquitin-conjugating

enzymes (E2), and ubiquitin-protein ligases (E3). First, an E1 activates Ub at the expense of ATP. Next, activated Ub is transferred to an E2 enzyme. Finally, an E3 specifically recognizes its protein substrate, which can be in its normal conformation or misfolded, and catalyzes the transfers of activated Ub from an E2 to the substrate. Successive addition of Ub to a lysine residue of a previously conjugated Ub results in the formation of a polyubiquitin chain. K48-linked polyubiquitin chains serve as a recognition signaling for proteasomal degradation. Once ubiquitinated substrates are transferred to the proteasome, DUBs remove the Ub chain, allowing for free Ub monomers to be recycled. Monoubiquitination and noncanonical polyubiquitination (e.g., K63 ubiquitin linkages) of proteins have been implicated in nonproteasomal cellular processes, including endocytosis, trafficking, cell signaling, DNA damage repair, and modifications of histones [17, 18].

UCH-L1 was first identified as a member of the ubiquitin carboxyl-terminal hydrolase (UCH) family of DUBs with cysteine protease activity in the late 1980s [1]. UCH-L1 is an abundant neuronal protein, comprising approximately 2% of total brain protein [1, 19]. Although low levels of UCH-L1 protein have been reported to be present in kidneys, breast epithelium, and reproductive tissues [20, 21], UCH-L1 is absent in most other tissues [1, 19, 22, 23]. UCH-L1 appears to play an important role in neurons, as mice lacking functional UCH-L1 have been reported to exhibit neuronal dysfunction and neurodegeneration [24, 25]. At the subcellular level, UCH-L1 is primarily found in the cytoplasm [26], but recent reports indicate that a subpopulation of UCH-L1 can be transiently localized to the nucleus [13, 27]. Biochemical studies revealed that UCH-L1 hydrolyzes Ub at its C-terminal glycine residue to generate monomeric Ub *in vitro* [1] and that this activity is dependent upon the catalytic residues C90 and H161 [2]. Analysis of UCH-L1 crystal structure indicates that these catalytic residues are not accessible to large polymers of Ub and suggest that UCH-L1 preferentially binds monomeric Ub and small adducts of Ub [28]. It is possible that substrate binding and/or the presence of cofactors may induce a conformational change, allowing UCH-L1 to process larger Ub chains. However, this has not yet been demonstrated *in vitro* or *in vivo*. Thus, UCH-L1 is best understood to function as a cysteine protease capable of hydrolyzing small Ub moieties.

Although the exact function of UCH-L1 is not fully understood, several studies suggest that UCH-L1 regulates the cellular pool of free Ub (Figure 1). First, UCH-L1 has been reported to cleave the ubiquitin gene products UbB and UbC and the ribosomal ubiquitin fusion protein UbA80 to generate monomeric Ub [29], leading to an increase in the level of free Ub (Figure 1). UCH-L1 may also elevate free Ub levels by facilitating recycling of Ub (Figure 1). Next, it has also been suggested that UCH-L1 plays a role in stabilizing Ub monomers by binding to monomeric Ub and preventing its lysosomal degradation (Figure 1) [4]. Association of UCH-L1 with monomeric Ub occurs independently of the catalytic C90 residue, indicating that mono-Ub binding is not dependent upon UCH-L1 hydrolase activity [4]. The role of UCH-L1 in the regulation of the free Ub pool is also supported by the observation that levels of monomeric Ub are decreased in gracile axonal dystrophy (*gad*) mice, which lack functional UCH-L1 [4] In contrast to other DUBs, *in vitro* studies indicate that UCH-L1 does not directly catalyze the deubiquitination of ubiquitinated protein substrates [29]. Moreover, no *in vivo* UCH-L1 substrates have been identified thus far. Collectively, current evidence suggests that UCH-L1 functions to increase the cellular pool of free Ub by hydrolyzing small Ub chains and stabilizing monomeric Ub rather than by directly acting on polyubiquitinated substrates.

UCH-L1 has been reported to possess putative, dimerization-dependent E3 ligase activity in addition to its hydrolase function (Figure 1) [5]. *In vitro* studies show that dimeric UCH-L1 promotes K63-linked polyubiquitination of α-synculein [5]. Unlike other E3 ligases, UCH-L1 E3 ligase activity was observed in the absence of ATP [5], which differs from the mechanism of conventional ubiquitination [17, 18]. It is currently not known whether UCH-L1 exhibits E3 ligase activity *in vivo*. Further investigation into UCH-L1 enzymatic function is needed to understand its role in health and disease.

3. UCH-L1 as a Positive Regulator of Tumorigenesis

Although UCH-L1 is almost exclusively expressed in neurons [1, 19], proteomic screens have revealed that UCH-L1 is present in many nonneuronal human tumors (Table 1) including adenocarcinoma [35], pancreatic ductal carcinoma [36], and squamous cell carcinoma [31]. Similarly, microarray profiling analyses show UCH-L1 mRNA is upregulated in several breast cancer tumor types [37] and medullary thyroid carcinoma tumors [38]. UCH-L1 mRNA has also been shown to be elevated in gall bladder and colorectal tumor tissues as a result of hypomethylation of the UCH-L1 promoter [39, 40]. High levels of UCH-L1 protein have also been observed in many human tumor-derived cell lines (Table 1) such as those cultured from lung [8], prostate [41, 42], and bladder tumors [43] as well as B-cell lymphomas [44] and osteosarcomas [45]. The presence of UCH-L1 in nonneuronal tumor tissues and cancer cell lines suggests that increased levels of UCH-L1 may promote oncogenic transformation and, therefore, point to a possible role for UCH-L1 as an oncogene in cancer pathogenesis.

The potential oncogenic function of UCH-L1 is supported by a number of clinical studies demonstrating that UCH-L1 expression level in tumors is inversely correlated with patient survivability [14, 36, 37]. High levels of UCH-L1 mRNA in breast tumors have been reported to be associated with poor prognosis in patients [37]. Likewise, elevated UCH-L1 mRNA in colorectal tumors is associated with higher incidence of tumor recurrence and shorter survival time [14]. Moreover, UCH-L1 expression in pancreatic ductal tumors is correlated with decreased patient survival [36]. Together, these data suggest that UCH-L1 is involved in tumor maturation.

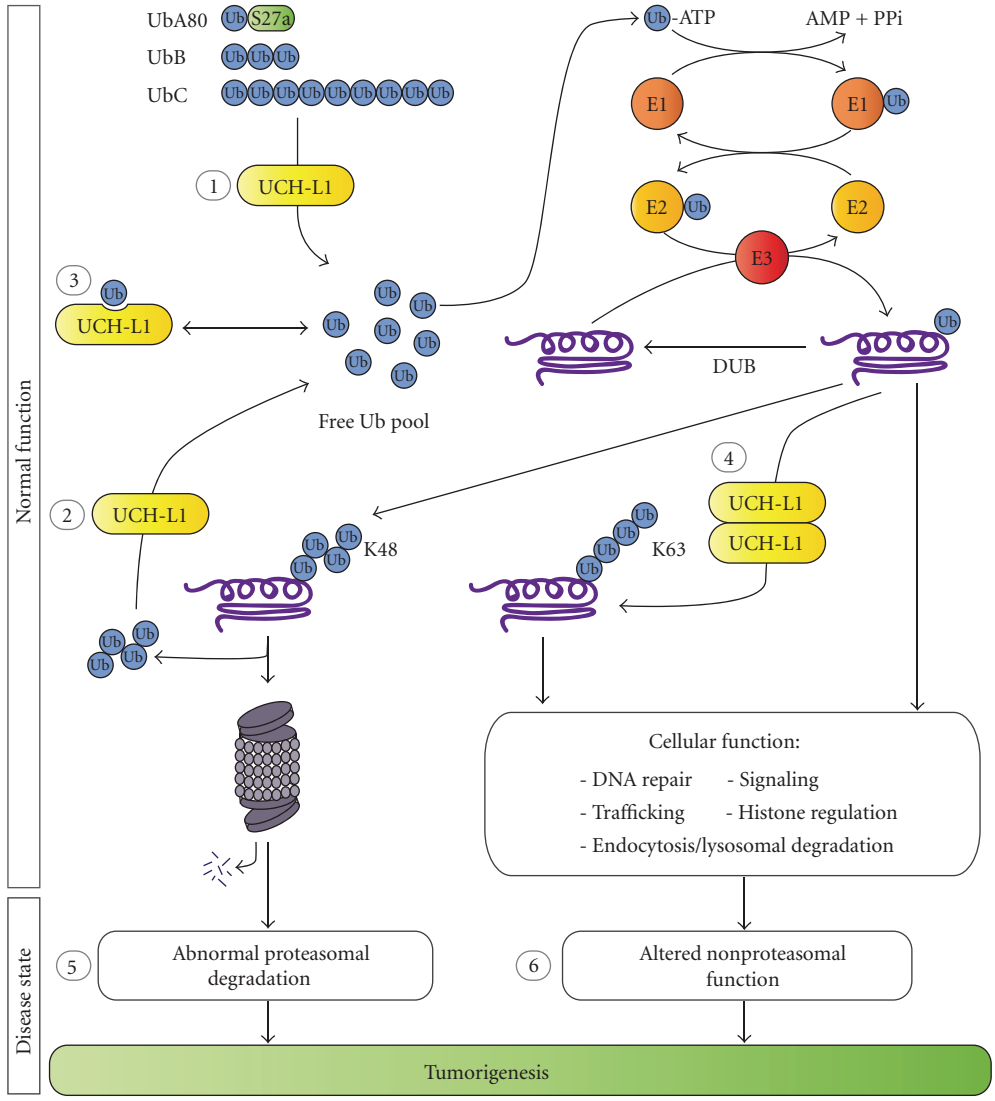

Figure 1: Molecular functions of ubiquitin c-terminal hydrolase L1. (1) UCH-L1 can hydrolyze ubiquitin pro-proteins to generate monomeric ubiquitin (Ub) [29]. (2) UCH-L1 may also facilitate Ub recycling by processing Ub chains. (3) UCH-L1 has been reported to stabilize monomeric Ub by binding to Ub and preventing its degradation by the lysosome [4]. Collectively, these functions (1, 2, and 3) give UCH-L1 control over the availability of free Ub and, therefore, the potential to influence many ubiquitination-dependent cellular processes, including proteasomal degradation, DNA damage repair, trafficking, cell signaling, endocytosis, and lysosomal degradation. (4) Dimerized UCH-L1 may possess ATP-independent E3 ligase activity that facilitates K63-linked polyubiquitination [5], although it is currently unclear whether this putative E3 ligase activity directly regulates ubiquitination of protein substrates *in vivo*. (5) Altered expression of UCH-L1 may cause changes to the free Ub pool, resulting in abnormal K48-linked polyubiquitination and proteasomal degradation. (6) Changes in the free Ub pool may also affect mono- and K63-linked polyubiquitination, leading to altered nonproteasomal functions and tumorigenesis.

To determine whether upregulation of UCH-L1 is a result of oncogenic transformation or itself a driving force of tumorigenesis, the direct involvement of UCH-L1 in cancer pathogenesis has been investigated. *In vitro* tumorigenesis studies show that UCH-L1 stimulates oncogenic transformation and invasion in nonsmall cell lung carcinoma [8] and colorectal cancer [14] cells, suggesting that UCH-L1 may function as an oncogene in these cancers. Furthermore, Hussain et al. have demonstrated that transgenic mice constitutively expressing UCH-L1 under the control of a CAGGS promoter form sporadic tumors in all tissues [11]. Of these tumors, lymphomas are the most prevalent [11]. Further investigation revealed that shRNA-mediated knock down of UCH-L1 in immortalized B cells decreased cell growth and viability, suggesting UCH-L1 promotes the development of lymphomas by inhibiting cell death and by stimulating proliferation [11]. Collectively, these data suggest UCH-L1 is a potent oncogene with the capacity to promote tumorigenesis in many different cell types.

Recently, it has been suggested that UCH-L1 promotes cancer cell motility and invasion, which may contribute to its oncogenic role. Overexpression of UCH-L1 in HCT8 colorectal cancer cells has been reported to enhance cell migration [9]. Additionally, Kim et al. have shown that

Table 1: Aberrant expression of UCH-L1 in tumor tissues and cancer cells.

Elevated UCH-L1	Down-Regulated UCH-L1
Malignant Tumors	
Squamous cell carcinoma [31]	Prostate tumors [46]
Medullary thyroid carcinoma tumors [38]	Primary breast cancer tumors [16]
Osteosarcoma [45]	Primary nasopharyngeal carcinoma [10]
Adenocarcinoma [35]	Colorectal carcinoma [47]
Metastatic colorectal cancer tumors [9]	Melanoma [48]
Breast cancer tumors [37]	Diffuse-type gastric cancer [34]
Pancreatic ductal carcinoma tumors [36]	
Parathyroid carcinoma [49]	
Transformed Cells	
SaOS-2 osteosarcoma cells [45]	LNCaP prostate cancer cells [50]
BLZ-211 and BLS-211 bladder cancer cells [43]	
BL30, X-50/7, KR4, Raji, KR4 B-cell lymphoma cells [44]	
HCT8 colorectal cancer cells [9]	
DU154 prostate cancer cells [41, 42]	
H157, W138, H358 lung carcinoma cells [8]	

siRNA-mediated knock down of UCH-L1 reduces H157 lung carcinoma cancer cells migration *in vitro* [8]. They further demonstrated that depletion of UCH-L1 attenuates lung metastasis *in vivo* in a murine xenograft model [8]. UCH-L1 stimulates prostate cancer cell migration and invasion as well by promoting epithelial-to-mesenchymal transition (EMT) [41]. UCH-L1 level also appears to be correlated with cancer cell metastatic capacity. While UCH-L1 is found in many lung carcinoma cell lines, it is further upregulated in high metastatic lines [8]. Likewise, low metastatic LNCaP and RWPE1 prostate cancer cells do not express UCH-L1, while high metastatic DU145 prostate cancer cells abundantly express UCH-L1 [41]. These studies suggest that UCH-L1 promotes cancer cell metastasis. Further studies are needed to determine how UCH-L1 regulates cell motility and invasion.

Despite growing evidence implicating UCH-L1 as a positive regulator of tumor growth and development, the mechanism by which UCH-L1 conveys oncogenesis is not fully understood. Many of the investigations into the role of UCH-L1 in cancer have focused on upregulation of UCH-L1 in tumor tissues and cancer cells. However, little is known about changes in UCH-L1 enzymatic activity during tumorigenesis. Although one group has observed a decrease UCH-L1 hydrolase activity in cervical carcinoma tissues and an increase in hydrolase activity in transformed keratinocytes [12], the role of UCH-L1 enzymatic function(s) in cancer is largely unknown. Furthermore, no evidence of genetic amplification of UCH-L1 or oncogenic mutations in UCH-L1 have been reported to date, although a Parkinson's disease-linked mutation has been identified [51]. Elucidation of UCH-L1 enzymatic activity in tumorigenesis and investigation into oncogenic genetic alterations of UCH-L1 may provide insights into the role of this enzyme in cancer pathogenesis.

4. UCH-L1 as a Potential Tumor Suppressor

In contrast to the body of literature identifying UCH-L1 as an oncogene, several reports have been put forth suggesting UCH-L1 acts as a tumor suppressor during the pathogenesis of certain cancers [10, 16, 46, 50]. Contrary to previous reports proposing UCH-L1 enhances the progression of prostate cancer [41, 42], two recent studies from Ummanni et al. suggest that UCH-L1 attenuates prostate tumor growth and maturation [46, 50]. UCH-L1 may possibly act as a tumor suppressor in breast cancer pathogenesis as well [16, 52]. In contrast to previous studies demonstrating that UCH-L1 is upregulated in breast tumors [21, 37], UCH-L1 mRNA expression was reported to be decreased in several breast carcinoma cell lines [16]. Moreover, ectopic expression of UCH-L1 in breast cancer cells caused a decrease in anchorage-independent cell growth and an increase apoptosis, suggesting UCH-L1 may act as a negative regulator of breast tumorigenesis [16, 52]. UCH-L1 has also been implicated in the suppression of nasopharyngeal carcinoma as UCH-L1 mRNA expression is decreased in many nasopharyngeal tumors [10]. Lastly, UCH-L1 promoter methylation is elevated in malignant prostate tumors [46], primary breast tumors [16], and nasopharyngeal carcinomas [10]. Similarly, several breast cancer [16] and gastric cancer cell lines [34] exhibit enhanced methylation of UCH-L1 promoter sequences, resulting in decreased UCH-L1 transcription (Table 1). Taken together, these observations suggest that UCH-L1 may function as a tumor suppressor, particularly in prostate [46, 50], breast [16, 52], and nasopharyngeal [10] carcinomas.

There are a number of possible reasons for the discrepancies in the observed oncogenic and tumor suppressor functions of UCH-L1 in tumorigenesis. First, studies suggesting UCH-L1 attenuates prostate cancer progression

[46, 50] focused on the behavior of low metastatic prostate cancer cells, while those implicating UCH-L1 as a positive regulator of prostate tumorigenesis [41, 42] investigated more mature prostate tumors and cell lines. Whether UCH-L1 elicits different effects as prostate tumors become more malignant remains to be investigated. Next, many studies have suggested UCH-L1 functions as a tumor suppressor based on observed decreases in UCH-L1 mRNA in tumor tissues and transformed cells [10, 16, 34, 47, 48]. However, it is not known whether differences in UCH-L1 transcription in these tumors and cells result in changes in UCH-L1 protein level and/or UCH-L1 enzymatic activity. Finally, UCH-L1 is absent or expressed at very low levels in all nonneuronal tissues [1, 19, 22, 23], raising an important question regarding the reported tumor suppressor role for UCH-L1: how can a reduction in UCH-L1 mRNA in tissues that normally express little to no UCH-L1 protein convey oncogenic transformation? To address this question, the normal expression pattern and/or physiological role of UCH-L1 in nonneuronal tissues need to be clarified.

5. Potential Mechanisms of Actions of UCH-L1 in Tumorigenesis

Currently, the precise mechanism(s) of UCH-L1-mediated tumorigenesis are not fully understood. Previous studies have identified several cancer-related signaling processes that are regulated by UCH-L1, which may contribute to its role in oncogenesis. In particular, UCH-L1 has been implicated in the regulation of cell cycle progression, cell survival, cell motility, and invasion (Figure 2).

5.1. UCH-L1 Enzymatic Activity and Oncogenesis. Disruption of UPS function has been implicated in cancer pathogenesis and progression [53] and many cancer-related cellular processes are controlled by ubiquitination, including cell division, growth factor signaling, DNA damage repair, and apoptosis [54–57]. As previously stated, UCH-L1 hydrolyzes small Ub molecules to generate free Ub and also stabilizes monomeric Ub [4, 29] (Figure 1). Through these functions, UCH-L1 can increase the free pool of Ub and, therefore, indirectly affect many ubiquitination-dependent cellular activities. In a pathogenic state, UCH-L1 dysfunction has the potential to alter the cellular levels of monomeric Ub, possibly causing global changes in protein ubiquitination. Therefore, aberrant UCH-L1 signaling may indirectly alter both the poly- and monoubiquitination of oncogenes and tumor suppressors, possibility leading to abnormal protein degradation and/or altered protein function and subsequent tumorigenesis (Figure 1).

UCH-L1 has also been reported to promote K63-linked polyubiquitination of α-synuclein through its putative, dimerization-dependent E3 ubiquitin ligase activity [5]. K63-linked polyubiquitination has been implicated in cancer-related cellular processes such as DNA damage repair and cell survival signaling [58, 59]. Although UCH-L1 E3 activity has not been observed *in vivo* and substrates other than α-synuclein have not been identified, alterations in UCH-L1 function may disrupt K63-linked polyubiquitination, possibly altering nonproteasomal cellular processes to promote tumorigenesis (Figure 1). Further investigation is needed to clarify the potential E3 function of UCH-L1 as well as the normal physiological and oncogenic role of this enzymatic function.

5.2. A Possible Function for UCH-L1 in Cell Cycle Regulation. UCH-L1 has been shown to stimulate proliferation in transformed lymphocytes and cervical carcinoma cells [11, 27], while it promotes G1/S arrest in breast cancer cells [16]. Together, these studies imply that UCH-L1 contributes to cancer pathogenesis by regulating cell division, although the exact control that UCH-L1 confers on the cell cycle remains unclear. UCH-L1 has been shown to modulate the levels of several cell cycle regulators in cancer cells including cyclin D [31] and p53 [10]. Coimmunoprecipitation experiments conducted by Caballero et al. have shown that UCH-L1 also interacts with JAB1 (Jun-activation domain-binding protein 1). Binding of UCH-L1 to JAB1 promotes the nuclear export and subsequent proteasomal degradation of the cyclin dependent kinase inhibitor p27 [13], resulting in increased cell proliferation (Figure 2(a)). These observations suggest UCH-L1 controls cell cycle progression by modulating the availability of cell cycle regulatory proteins, possibly by altering their ubiquitination status. Recent evidence implicates UCH-L1 in the regulation of cell cycle progression via direct interactions with microtubules. Bheda et al. have demonstrated that UCH-L1 is tightly associated with microtubules during cell division in several transformed cell lines, and that siRNA-mediated knockdown of UCH-L1 reduces microtubule assembly and disassembly [27]. Interestingly, both 25 kDa and 50 kDa UCH-L1 species were associated with purified microtubules [27], suggesting UCH-L1 may act as a dimer to regulate microtubule dynamics. Taken together, these observations suggest that UCH-L1 regulates cell cycle progression by altering levels of cell cycle regulatory proteins and by controlling microtubule dynamics. However, further studies are needed to determine the specific manner by which UCH-L1 controls cell division. In particular, whether or not UCH-L1 controls ubiquitination of cell cycle regulators should be examined.

5.3. UCH-L1 and Cell Survival Signaling. Overactivation of the serine-threonine kinase Akt is a common hallmark of cancer pathogenesis [60]. Phosphorylation of Akt leads to activation of several signaling cascades that together promote cell survival by stimulating proliferation and inhibiting apoptosis. Pharmacological inhibitors of Akt kinase activity attenuate UCH-L1-mediated ECM invasion in nonsmall cell lung carcinoma cells [8]. Additionally, overexpression of UCH-L1 in these cells increases phosphorylation of the downstream Akt targets p38 and ERK1/2, suggesting that UCH-L1 promotes cell survival through Akt-dependent activation of MAPK signaling [8]. Lastly, overexpression of UCH-L1 in immortalized B cells also has been shown to increase Akt phosphorylation during lymphoma progression [11]. Together, these data suggest UCH-L1 elicits at least

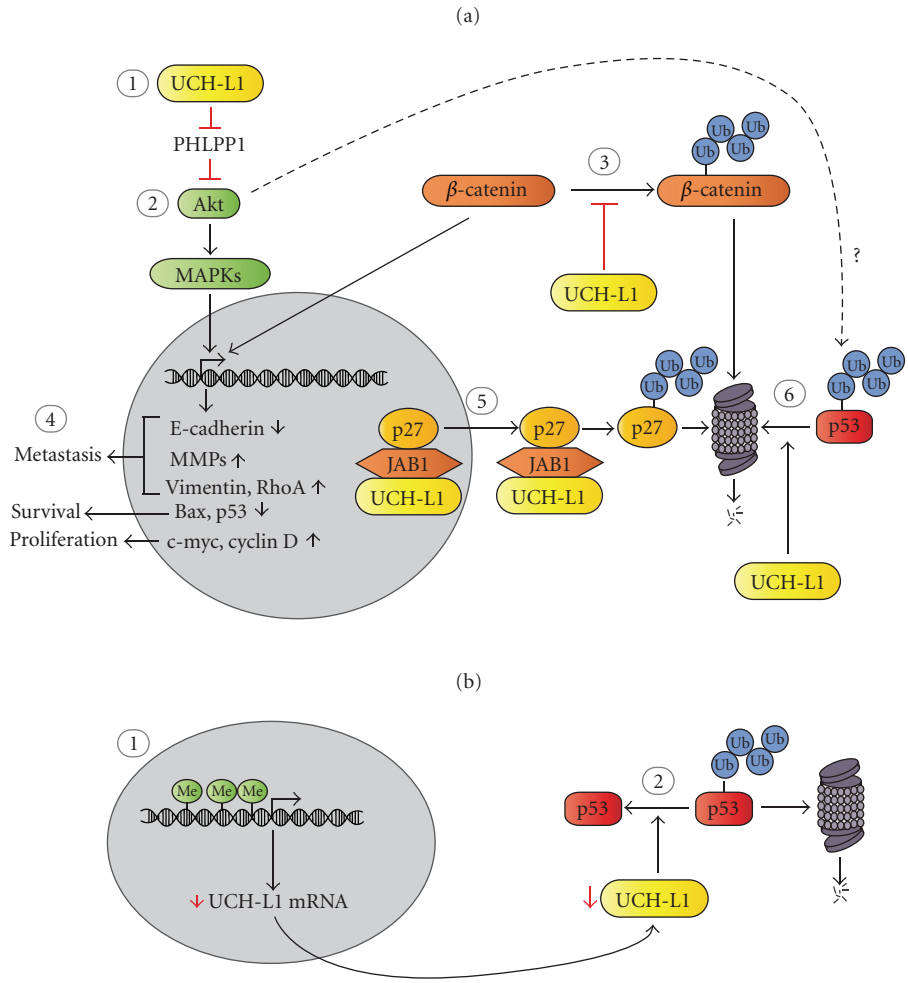

Figure 2: The potential roles of UCH-L1 in tumorigenesis. (a) UCH-L1 as a possible oncogene that promotes metastasis and cell growth. (1) UCH-L1 is up-regulated in several tumor tissues and cancer cell lines [7–13]. (2) Elevated UCH-L1 may stimulate Akt through inhibition of the phosphatase PHLLP1 [11], leading to increased MAPK signaling [8]. (3) UCH-L1 has been reported to decrease polyubiquitination and proteasomal degradation of β-catenin, resulting in enhanced β-catenin-mediated transcription [30]. (4) Increased β-catenin and Akt signaling could potentially cause changes in gene transcription that promote metastasis and proliferation and inhibit apoptosis, resulting in enhanced oncogenicity [31–33]. (5) UCH-L1 binds to JAB1 and promotes the nuclear export and subsequent proteasomal degradation of the cell cycle inhibitor p27 [13]. (6) Upregulation of UCH-L1 has been reported to promote proteasomal degradation of p53 [11], which may be a consequence of activation of Akt signaling. Reduction of p27 and p53 levels by UCH-L1 may attenuate cell cycle arrest, allowing for uncontrolled cell growth. (b) UCH-L1 as a putative tumor suppressor in certain cancer subtypes. (1) Reduction of UCH-L1 transcription via promoter methylation-silencing has been observed in certain cancer cells and tumor tissues (e.g., nasopharyngeal carcinomas [10] and gastric cancer cells [34]). (2) In these cancer types, it has been proposed that UCH-L1 promotes deubiquitination of p53 and inhibits its proteasomal degradation [10, 16]. Reduced UCH-L1 transcription due to promoter methylation may thus lead to increased degradation of p53, resulting in a reduction of p53-mediated transcription of tumor suppressing genes and enhanced tumorigenesis (see text for more details).

some of its cellular effects through Akt-dependent signaling and that stimulation of Akt by UCH-L1 is a potential mechanism of UCH-L1-mediated oncogenesis (Figure 2(a)). UCH-L1 promotes Akt signaling, in part, by reducing the level of the tumor suppressor PHLPP1 [11], a phosphatase that reverses Akt phosphorylation rendering Akt inactive. However, the mechanism by which UCH-L1 suppresses PHLPP1 merits further investigation as UCH-L1 does not alter PHLPP1 transcription or promote proteasomal degradation of PHLPP1. Furthermore, whether or not UCH-L1 modulates upstream activators of Akt remains to be determined. Nevertheless, stimulation of Akt by UCH-L1 and the subsequent promotion of prosurvival signaling may contribute to the function of UCH-L1 in oncogenesis.

A number of studies suggest that UCH-L1 exerts its actions through regulation of the tumor suppressor p53. However, the specific manner in which UCH-L1 modulates p53 level and function remains controversial. UCH-L1 has been shown to promote the proteasomal degradation of p53 in HeLa cells [11], and microarray analyses conducted by Bheda et al. show that depletion of UCH-L1 in 293T cells increases the levels of many p53 target genes [32],

suggesting UCH-L1 suppresses p53 signaling. On the other hand, overexpression of UCH-L1 was reported to increase p53 levels in MDA-MB-231 breast carcinoma cells [16] and HONE1 nasopharyngeal carcinoma cells [10]. Similarly, Li et al. have shown that over-expression of UCH-L1 in LNCaP prostate cancer cells reduces polyubiquitination of p53, leading to inhibition of degradation of p53 by the proteasome [50]. They also observed an increase in polyubiquitination and degradation of mdm2 in response to UCH-L1 over-expression, suggesting UCH-L1 suppresses mdm2 to stabilize p53 levels [50].

Further studies are needed to determine specifically how UCH-L1 modulates p53. Discrepancies in observed regulation of p53 by UCH-L1 may be attributed to differences in p53 status. Studies implicating UCH-L1 as a negative regulator of p53 [11, 32] were conducted in cells with wild-type p53 [61, 62]. On the contrary, UCH-L1 elevates p53 levels in MDA-MB-231 and HONE1 cells, which express DNA binding domain mutant p53 with little to no transcriptional activity [63–65] and LNCaP cells, which have also been reported to express DNA binding domain mutant p53 [66], although this is controversial [67].This suggests that UCH-L1 may regulate wild-type and DNA binding domain mutant p53 differently. P53 is frequently mutated in human cancers [63] and variation in p53 status may offer another possible explanation for why UCH-L1 has been reported to function as an oncogene and a tumor suppressor in different cancer cell lines and tumor types. It is possible that in cells with wild type p53, UCH-L1 promotes degradation p53, resulting in reduced p53 signaling and inhibition of cell death (Figure 2(a)). On the other hand, in other cell types with weakened p53 transcriptional activity, UCH-L1 may regulate nontranscriptional functions of p53 [68, 69] to promote apoptosis and attenuation of tumor growth (Figure 2(b)). It is also possible that UCH-L1 indirectly elicits control over p53 by modulating negative regulators of p53, such as mdm2, as suggested by Li et al. [10]. As p53 level and function are regulated, in part, by ubiquitination [58, 70, 71], investigation into modulation of p53 ubiquitination by UCH-L1 may offer additional insights into the role of UCH-L1 in tumorigenesis. However, while exploring the relationship between UCH-L1 and p53 ubiquitination, it is important to keep in mind that, despite hypotheses to the contrary [10, 16], it is unlikely that UCH-L1 directly deubiquitinates or ubiquitinates p53 based on what is known about UCH-L1 structure and function [28, 29]. Additionally, it might be possible that activation of Akt signaling by UCH-L1 [8, 11] might also contribute to its control over p53, as Akt is an established negative regulator of p53 activity [60, 72].

5.4. The Potential Role of UCH-L1 in Metastasis. UCH-L1 has been suggested to promote metastasis in colorectal, lung, and prostate cancer cells [8, 9, 41]. Cancer cell metastasis is often attributed to hyperactivation of β-catenin, a transcription factor that when over-activated promotes cell migration and invasion [73]. UCH-L1 overexpression has been shown decrease polyubiquitination and proteasomal degradation of β-catenin in HEK 293 cells, leading to stabilization of TCF: β-catenin complexes and increased β-catenin-mediated transcription of prosurvival genes such as c-myc, c-jun, and survivin [30]. Although, it is unlikely UCH-L1 directly deubiquitinates β-catenin [28, 29], these observations suggest UCH-L1 may convey its oncogenic function through Wnt signaling pathways. UCH-L1 itself has been identified as a target of β-catenin-mediated transcription, suggesting there is a positive feedback loop between β-catenin and UCH-L1 that enhances metastasis [30]. One consequence of β-catenin signaling is promotion of epithelial-to-mesenchymal transition (EMT) [33]. Recently, it was shown that UCH-L1 enhances prostate cancer cell metastasis by increasing the expression of pro-EMT genes such as vimentin and matrix metalloproteinases (MMPs) and reducing transcription of the EMT suppressor E-cadherin [41]. Together, these data imply that UCH-L1 promotes cancer cell metastasis via β-catenin-induced EMT (Figure 2(a)). Therefore, therapeutic targeting of Wnt and EMT signaling may prove to be an effective treatment for tumors that express high levels of UCH-L1.

6. Conclusions

In summary, emerging evidence suggests that UCH-L1 is a potent oncogene that promotes tumor growth and development during the progression of many forms of cancer. However, the exact role of UCH-L1 in oncogenesis remains controversial, as UCH-L1 has been suggested to function as a tumor suppressor in certain tumor types. The observed involvement of UCH-L1 in the regulation of cell cycle progression, cell survival, and metastasis may explain its oncogenic role. However, further studies are needed to clarify the exact mechanisms of action of UCH-L1 in tumorigenesis. Continued investigation into the function of UCH-L1 in cancer may tell us whether or not UCH-L1 can be used as a diagnostic marker. UCH-L1 is upregulated in many cancer tissues and, therefore, high levels of UCH-L1, particularly in nonneuronal tissues, may serve as an early detection biomarker for tumors. Furthermore, UCH-L1 itself could be a potential therapeutic target, which may have benefits for the treatment of cancer. Elucidation of the role of UCH-L1 in cancer may lead to a better understanding of the molecular pathogenesis of tumors as well as potentially facilitate the development of novel cancer therapeutics and diagnostics tools.

Abbreviations

UCH-L1: Ubiquitin C-terminal hydrolase L1
Ub: Ubiquitin
UPS: Ubiquitin-proteasome system
DUB: Deubiquitinating enzyme
E1: E1 Ubiquitin-activating enzyme
E2: E2 Ubiquitin-conjugating enzyme
E3: E3 Ubiquitin-protein ligase
Akt: Protein Kinase B
MAPKs: Mitogen-activated protein kinases
ERK1/2: Extracellular signal-related kinases 1 and 2

PHLPP1: PH domain leucine-rich repeat protein phosphatase 1
JAB1: Jun-activation domain-binding protein 1
EMT: Epithelial-to-mesenchymal transition
MMPs: Matrix metalloproteinases.

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