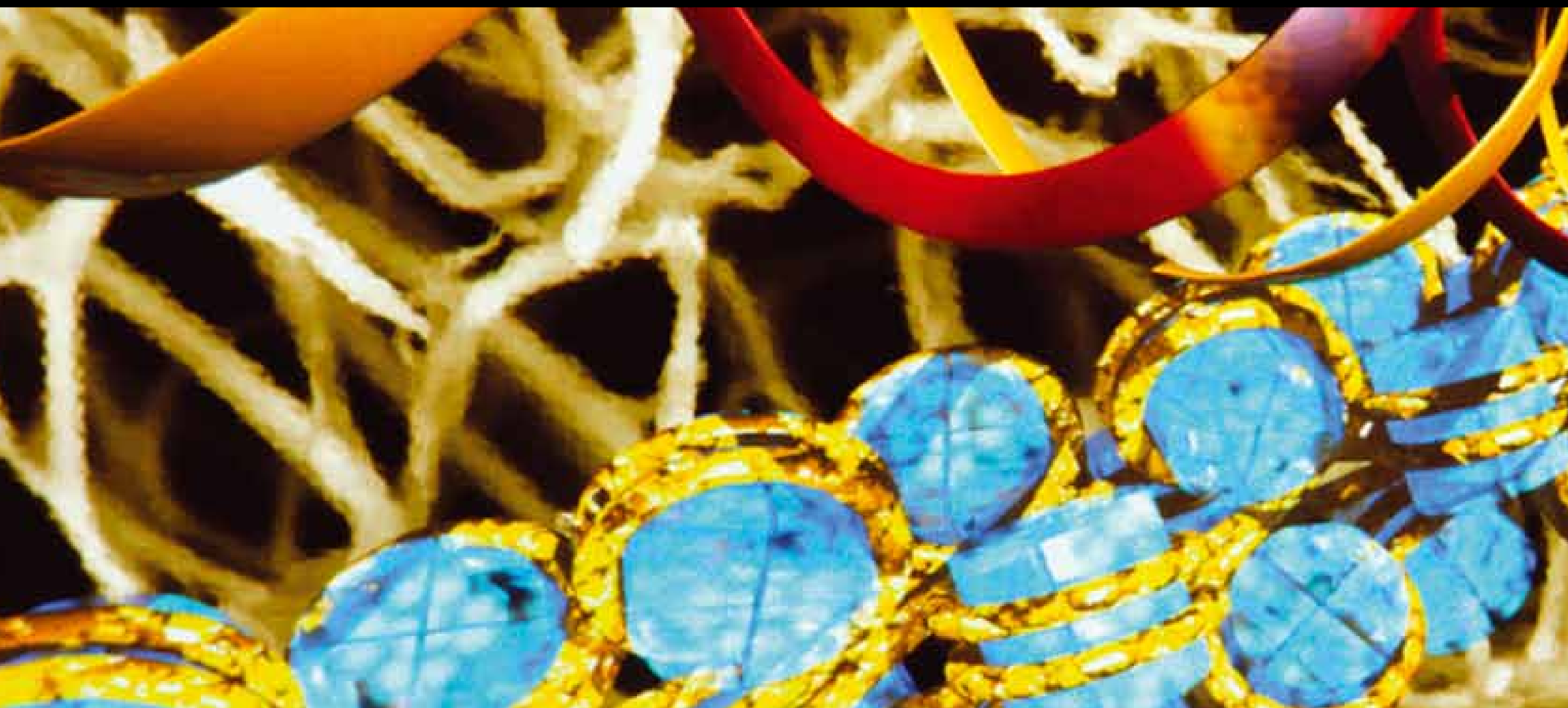
Molecular Biology International

RASSF Family Proteins

Guest Editors: Geoffrey J. Clark, Shairaz Baksh, Farida Latif, and Dae-Sik Lim



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Editorial

RASSF Family Proteins

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1. RASSF Family Proteins

The Ras-association domain family (RASSF) proteins are tumor suppressor proteins whose importance to the development of cancer has become increasingly apparent over the last 12 years. While possessing no enzymatic activity, they appear to function as scaffolding molecules to regulate the activity of a surprisingly broad array of effectors. They are implicated in the regulation of a diverse range of biological functions including apoptosis, autophagy, cell cycle control, microtubule dynamics, and DNA repair. In addition, they are thought to be one of the regulators of the Hippo pathway, the newly emerging tumor suppressor pathway evolutionarily conserved between *Drosophila* and mammals.

Typically, inactivation of RASSF genes in cancer involves epigenetic silencing of their promoters. Indeed, the RASSF1A promoter appears to be the most frequently inactivated promoter yet detected in human tumors. As PCR-based assays may be used to detect specific promoter methylation in body fluids with exquisite sensitivity, it may be possible to use the epigenetic inactivation of RASSF genes as prognostic/diagnostic markers. In this issue, inactivation of RASSF genes in hepatocellular carcinoma and bladder cancer is considered by D. F. Calvisi et al. and W. Meng et al. as examples.

The RASSF1A gene may be unique in the family as it also found to be frequently mutated in cancer cells. These mutants may provide useful tools for structure/function studies. Moreover, RASSF1A exhibits a polymorphism which is common in Caucasians and is associated with an enhanced risk of cancer. M. Gordon et al. include a comprehensive review of the current knowledge regarding RASSF1A mutants and polymorphisms.

RASSF1A is the best-characterized member of the RASSF family and most of our knowledge regarding the biological function of these proteins derives from studies with RASSF1A. It can modulate at least two major apoptotic signaling pathways. One via a modulator of apoptosis (MOAP-1)/BAX pathway, and the other via the intriguing and perplexingly complex hippo pathway. The interaction with the Hippo pathway may occur at multiple levels and may permit RASSF proteins to modulate cell cycle effects as well as apoptosis. J. Law et al. describe our current understanding of the MOAP-1 pathway while F. Fausti et al. and A. M. Richter et al. elaborate on the role of the Hippo and RASSF/Hippo connection in regulation of several aspects of biology.

RASSF1A may also impact the cell cycle and genetic stability by the modulation of microtubule dynamics. RASSF1A binds several microtubule-associated proteins (MAPs) directly and may use these to modulate microtubule dynamics essential to motility and spindle formation. This function may have therapeutic ramifications by influencing the sensitivity of tumor cells to microtubule-targeting cancer therapeutic agents such as Taxol, as described here by S. Kassler et al. A key role for RASSF1A has also been identified in modulating the DNA damage response. These aspects of RASSF1A are considered by S. F. Scrace and E. O'Neill. Thus again, the RASSF1A status of a cell may influence

the response to a therapeutic intervention, in this case, DNA damaging agents. A similar situation arises with the family member RASSF2, as described by J. Clark et al. The potential for epigenetic therapy to reverse the loss of function of RASSF genes is plausible and may enhance therapeutic approaches.

Although RASSF proteins are primarily regarded as tumor suppressors, there is now strong evidence that they may also play a key role in cardiac function. In particular, RASSF1A is important for hippo pathway driven cardiac hypertrophy responses. D. P. Del Re and J. Sadoshima add a review of the cardiac role of RASSF1A to complete the Special Issue.

The field has been catalyzed by an inaugural international RASSF symposium in 2009 in Banff, AB, Canada and a second meeting in Oxford, England in 2011. A third meeting is being planned for 2013 (RASSF Symposia Information at http://rassfsymposia.com/). These meetings brought together scientists and clinicians from all around the globe to share information and debate results. In the coming years, we anticipate more revelations demonstrating the biological importance of RASSF family proteins in human development and disease.

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Review Article

Hippo and *rassf1a* Pathways: A Growing Affair

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First discovered in *Drosophila*, the Hippo pathway regulates the size and shape of organ development. Its discovery and study have helped to address longstanding questions in developmental biology. Central to this pathway is a kinase cascade leading from the tumor suppressor Hippo (Mst1 and Mst2 in mammals) to the Yki protein (YAP and TAZ in mammals), a transcriptional coactivator of target genes involved in cell proliferation, survival, and apoptosis. A dysfunction of the Hippo pathway activity is frequently detected in human cancers. Recent studies have highlighted that the Hippo pathway may play an important role in tissue homoeostasis through the regulation of stem cells, cell differentiation, and tissue regeneration. Recently, the impact of RASSF proteins on Hippo signaling potentiating its proapoptotic activity has been addressed, thus, providing further evidence for Hippo's key role in mammalian tumorigenesis as well as other important diseases.

1. Introduction

The Hippo pathway is a signaling pathway that regulates cell growth and cell death. It was discovered in *Drosophila melanogaster* as a pathway controlling organ size and of which mutations lead to tumorigenesis. This pathway is highly conserved, and its activation or repression could lead to the following most extreme outcomes: proliferation/transformation and death/tumor suppression. The Hippo pathway cross-talks with other signaling players such as Notch, Wnt, and Sonic hedgehog (Shh). It influences several biological events, and its dysfunction may possibly lie behind many human cancers. In this review, we discuss the complex data reported about *Drosophila* to date (schematic representation in Figure 1) and the human Hippo (schematic representation in Figure 2) pathways focusing on the relationship between the tumor suppression *rassf* protein family and the Hippo-like pathway in humans [1, 2].

2. The Hippo Signaling Network in *Drosophila*

Drosophila imaginal discs have facilitated molecular dissecting of signaling pathways controlling organ size during development. These imaginal discs allow to screen how organs grow several folds larger before differentiating into adult organs after proliferation in larval stages. By using the genetic analysis in *Drosophila*, Robin W. Justice and colleagues were the first to describe that loss of Wts (Warts), which encodes a kinase of Nuclear Dbf-2-related (NDR) family, results in a *Drosophila* phenotype characterized by tissue overgrowth [3]. Several years later many components of this pathway were characterized. Four tumor suppressors called Hippo (Hpo), Warts (Wts), Salvador (Sav), and Mats were established. These suppressors constitute the core linear kinase cassette of Hippo/Warts pathway whose products can affect proliferation without increasing apoptosis susceptibility [3–6] (Figure 1). Subsequent genetic screens identified at

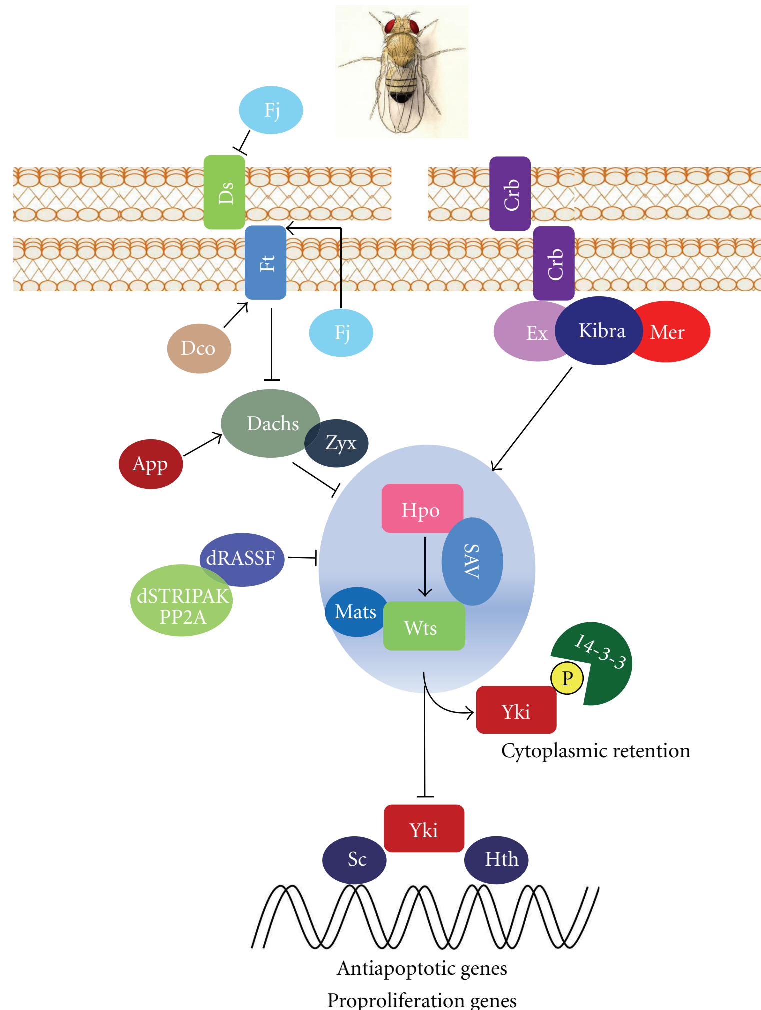

Figure 1: "Hpo signaling pathway in *Drosophila.*" Schematic representation of Hippo kinases cascade and of its modulation by apical transmenbrame protein complexes.

least seven additional tumor suppressors whose biological functions converge on Hpo and/or Wts: the FERM domain proteins Merlin (Mer) and Expanded (Ex) [7–10], the protocadherins Fat (Ft) [11–14] and Dachsous (Ds) [15, 16], the CK1 family kinase Disc overgrown (Dco) [17, 18], the WW and C2 domain-containing protein Kibra [19–21], and the apical transmembrane protein Crumbs (Crb) [22–24]. All of these suppressors converge and act through a common downstream component, the transcriptional co-activator protein Yorkie (Yki) [25] (Figure 1). The mechanisms by which these upstream regulators signal towards the final player Yorkie are complex and are still focus of investigation. A great deal of evidence suggests that they work in a combinatorial or synergistic manner to regulate Hippo kinase activity.

2.1. The Apical Protein Complex: Kibra, Expanded, and Merlin. The molecular link between upstream regulators and the core complex has not yet been clarified in mammals nor in *Drosophila.* In 2006, Hamaratoglu and collaborators proposed Mer (Merlin) and Ex (Expanded) as potential upstream regulators of the Hippo pathway [9], proteins which contain a FERM (4.1/ezrin/radixin/moesin) domain. Both proteins are considered tumor suppressors which cooperate to control organ growth. Their function seems to be partially redundant. In fact, while single mutation of each gene results in increased tissue growth, mutations in both genes give rise to a more strongly affected phenotype [9, 10]. Kibra, a third component of this apical complex, has recently been found. This protein possesses a WW domain which facilitates the interaction with other members of the Hippo pathway, such as Wts. It further interacts with a C2 domain that consists of a phospholipid-binding motif through which Kibra is believed to potentiate its membrane association [19–21]. WW domains are 35–40 amino acid protein–protein interaction domains that are characterized by a pair of conserved Trp residues, which generally interact with Pro-rich sequence motifs [26]. WW domain-Pro motif interactions appear to be particularly common in the Hpo

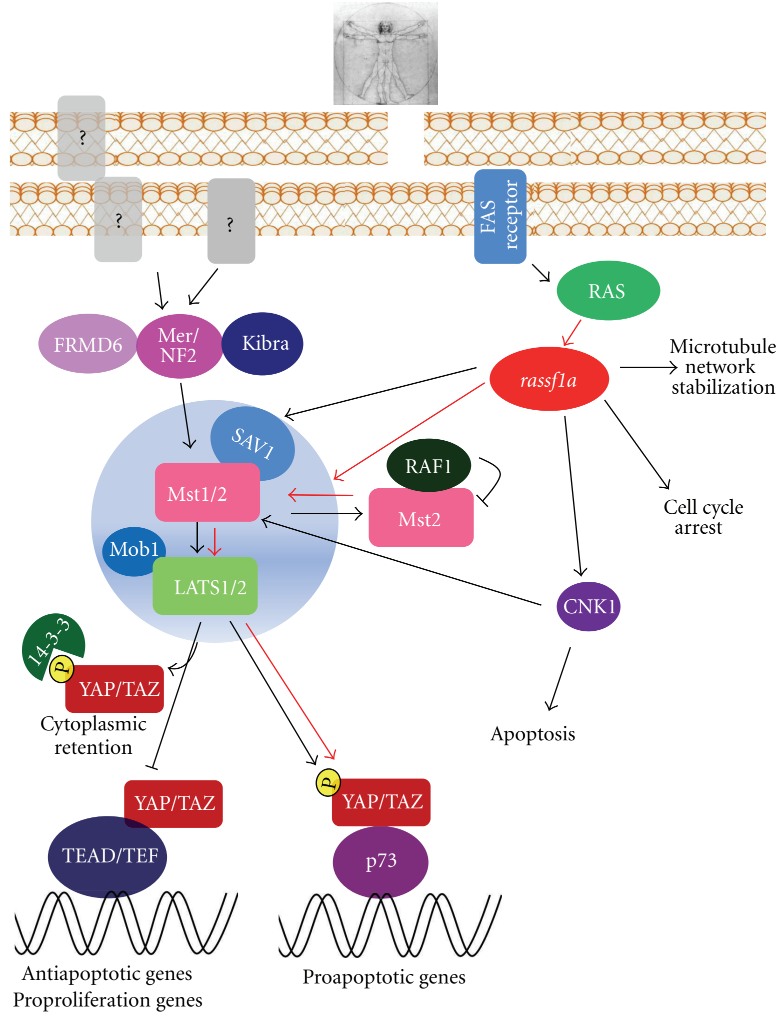

Figure 2: "Hpo signaling pathway in Mammals and the cross-talk with *rassf1a* signaling." Schematic representation of mammalian Hippo kinases cascade and interconnections between Hippo pathway and *rassf1a* protein signal. Red lines indicate the impact of *rassf1a* signaling in modulating activity of Hpo pathway components.

pathway. Three core components of Hpo signaling (Yki, Kibra, and Sav) contain WW domains, whereas three other components (Wts, Ex, and Hpo) hold PPxY motifs (reviewed in [27, 28]). While the formation of a ternary complex between Kibra, Ex, and Mer was observed, each protein was seen to localize to cellular membranes independently. Furthermore, it has been published that the Kibra-Mer-Ex complex is physically involved with the Hpo-Sav, constituting an apical protein complex required for associating the Hpo pathway to the cellular membranes [20, 21]. Studies on the Ex localization and function have led to the discovery of another important upstream regulator protein of Hpo, Crb (Crumbs) [22–24]. Crb is a transmembrane protein which normally localizes to the subapical membrane of epithelial cells that is responsible together with other apical complexes in *Drosophila* for organizing apical-basal polarity [29]. Crb binds to Ex through a short intracellular domain including a juxtamembrane FERM-binding motif (FBM). The FBM domain of Crb interacts with the FERM domain of Ex. This type of binding is necessary for Ex apical localization and stability. Furthermore, it has been published that Crb also works with Mer and Kibra [23]. The loss of Crb expression was shown to further determine a phenotype characterized by overgrowth, possibly to a lesser degree compared to the other members of Hpo signaling described until now [22–24]. Not long ago, this protein was proposed to have had an important function as a transmembrane receptor recognizing cell-cell contacts through Crb-Crb binding domains [22].

2.2. The Upstream Regulator: Transmembrane Protein Fat. The atypical cadherin FAT (Ft) was the first transmembrane protein shown to affect Hippo signaling. Fat is the first tumor suppressor gene isolated in *Drosophila*. In fact, the complete knock-out of the FAT protein induces death in *Drosophila* larvae with overgrown imaginal discs [11]. As previously mentioned, FAT is a large transmembrane protein, constitutively cleaved by unknown proteases. It contains

34 cadherin repeats in its extracellular domain, functioning as a receptor for Hippo signaling [12–14] as well as for planar cell polarity (PCP) [30, 31]. PCP is a mechanism through which cells orient themselves orthogonally to the apical-basal axis, as observed in the wing hairs of *Drosophila*, and the sensory hair cells in the inner ear of mouse. Notably, the mechanism by which FAT regulates Hippo signaling is different from the branch involving the ternary complex Ex-Mer-Kibra. Many lines of evidence suggest that the principal mechanism exerted by FAT is on the Wts function [18, 32]. Thus, FAT-Hpo signaling is genetically distinguishable, involved in Hippo pathway regulation of imaginal discs and neuroepithelial tissue, but not in other tissues such as ovarian tissue [14, 33, 34]. Many genes were reported to take part in this parallel mechanism together with FAT. First, Dachsous (Ds), an atypical cadherin which binds to FAT [15, 16]. FAT is regulated by an expression gradient of Ds [35, 36]. Four-jointed (Fj) is a kinase that typically localizes to the Golgi subcellular compartment and that phosphorylates the cadherin domains of FAT and Ds to mediate binding between these two proteins [37]. Another kinase responsible for FAT phosphorylation in its cytoplasmatic segment is a Casein I kinase, termed Discs overgrown (Dco) [17, 18]. The effective key mediator of FAT in the Hippo pathway seems to be Dachs, an unconventional myosin which antagonizes FAT, and whose activity is influenced by Approximated (App) [17]. App, in fact, antagonizes FAT signaling by modulating Dasch expression [38]. Another protein identified recently linked to the FAT branch in Hippo signaling is the LIM-domain protein Zyx102. It has been found to directly affect the core kinases of the Hippo pathway [39]. All of these components described above seem to be responsible for linking Hippo to extracellular stimuli [40].

Another so called "scaffold" protein that has been identified as a regulator of Hpo is called *Drosophila rassf* (*drassf*). This protein like its mammalian counterpart *rassf* can bind to Hpo through a conserved SARAH domain. But unlike in mammals, it hampers Hpo activity by competing with SAV to bind to Hpo [41] and by recruiting a Hpo-inactivating PP2A complex (dSTRIPAK) [42], thus showing a positive regulation of growth. Interestingly, Grzeschik and collaborators showed that the depletion of the *Drosophila* neoplastic tumor suppressor Lethal giant larvae (Lgl), which controls apical-basal cell polarity and proliferation, leads to upregulation of the Hippo pathway target Yki through a decreased phosphorylation and consecutively overproliferation of developing eyes, without affecting apical-basal polarity [43]. This mechanism is brought about by cellular mislocalization of Hpo and *rassf*. These both colocalize basolaterally leading to the deregulation of the Hippo kinase cascade, thereby preventing phosphorylation and inactivation of Yki. This concurs with data previously discussed wherein *rassf* is able to bind to Hpo precluding its interaction with SAV [41].

2.3. The Key Effectors of Growth Control: Hippo, Warts, Salvador, and Yorkie. Warts is crucial in the phosphorylation-dependent regulation of Yki [25, 44, 45]. Warts (Wts) encodes a Ser/Thr kinase of Nuclear Dbf-2-related (NDR) family. The activity of Warts is controlled through a series of phosphorylation events. Warts is directly phosphorylated by Hippo (Hpo), a member of the Sterile-20 family of Ser/Thr kinases, in a reaction that is facilitated by the Salvador protein [4, 5]. The fly protein Hippo (Hpo) is the first mediator of this pathway characterized by a kinase cascade. Wu and collaborators identified Hpo through analysing the phenotype of *Drosophila* Hpo mutants. Hpo is a kinase protein that regulates cell proliferation as well as apoptosis in *Drosophila*. In addition, it interacts, phosphorylates, and is activated by the WW domain-containing protein Salvador. Salvador (Sav) was described as a tumor suppressor gene, whose loss caused tissue overgrowth, similar to Wts loss of function. Tapon and collaborators were the first to observe, in 2002, that loss of Sav or Wts was strictly associated with increased expression of *cyc e*, a cell cycle progression regulator and *diap1*, an apoptosis inhibitor, thus, confirming these that two proteins' very important role in coordinating these two cellular processes [4]. Similar to Sav function on Hpo, Mats' role (Mob as tumor suppressor) which also belongs to the NDR family, as well as its kinase-like behavior binding to and potentiating Wts intrinsic activity, was described in 2005 [6]. Thus, Sav and Mats action as adaptor proteins, often termed scaffold proteins, both serve to potentiate Hippo signaling. Interestingly, it was also reported that Mats is a Hpo substrate. The latter phosphorylates Mats increasing its affinity for Wts binding, thus inducing potentiation of Wts kinase activity [46].

The downstream key regulator of Hpo signaling is Yorkie (Yki). It was identified in a yeast two-hybrid screen for Wts-binding protein, which is the final step in the Hippo pathway, driving its transcriptional regulation [25]. Yki is not a direct transcriptional factor because it does not possess its own consensus DNA-binding motif but is known as a potent transcriptional co-activator by cooperating with different DNA-binding proteins. Wts directly phosphorylates Yki at Ser 168, thus creating a binding site for 14-3-3 proteins which sequester Yki in the cytoplasm and prevent its nuclear import [44, 45]. In actual fact, the loss of Hippo signaling as well as mutations in 14-3-3 binding site for Yki was shown to produce strong nuclear accumulation, a common feature, coupled with aberrant activity of Yki [47]. Another two residues of Yki are believed to be targets of Wts phosphorylation (Ser111 and Ser250); however, little is known about the underlying mechanisms. As mentioned before, Yki cooperates with many DNA-binding proteins which act as transcription factors, potentiating their function. It is worth noting that some binding partners of Yki are the same kinases that function upstream to it in the Hippo pathway. Thus, through the PY (PPxY)-WW domain interactions, Yki is able to bind to Ex, Wts, and Hpo that sequester Yki at a cytoplasmatic level, independently from its phopshorylated state [48, 49]. Loss of Hippo signaling and consecutive aberrant Yki activation leads to deregulation of some gene class transcriptions. One class includes genes involved in cell survival and proliferation. One of the Yki partners, Scalloped (Sc), a member of TEAD/TEFs family, is responsible for Yki overexpression induced tissue

overgrowth [50, 51]. Another partner of Yki in *Drosophila* is Homothorax (Hth) that promotes cell survival and cell proliferation in eye development from eye imaginal discs [52]. Both Sc and Hth are able to bind a Hippo consensus DNA motif, termed Hippo response element (HRE), which is present in many Hippo target genes. Particularly, Sc together with Yki bind to the HRE present in a very well-known target gene, *diap1* [50], an apoptosis inhibitor, as mentioned above. Hth has only little influence on *diap1* transcription. It is very important in regulating the transcription of another Yki target, the growth promoting microRNA gene *bantam*. Other Yki targets in this class are the cell-cycle regulators *cyc e*, *e2f1* [4, 53], and *Drosophila* Myc (dMyc) whose expression seems to be positively regulated by Yki [54, 55]. Another important class is made up of components from other signaling pathways, such as ligands for Notch, Wnt, EGFR, and Jak-Stat pathways. In fact, other known Yki partners are believed to be Smad proteins [56]. This interaction appears to potentiate the transcriptional response to BMP/TGF-β signaling, addressing a possible crosstalk between Hippo and BMP/TGF-β pathway. Finally, a third class of Yki targets consisting of several proteins from its own Hippo cascade, such as Ex, Mer, Kibra, Crb, and Fj. These are downstream transcriptional targets of Yki [9, 17, 20, 57] and define a sort of positive feedback loop which characterizes most signal pathways.

3. The Hippo Kinase Signaling in Mammals

3.1. YAP and TAZ: Mammalian Effectors of Hippo Pathway. The Hippo pathway is highly conserved in mammalian systems. It was demonstrated that loss of function of mutant flies can be rescued by expressing their respective human counterparts [5, 6]. These data strongly correlate with the importance of Hippo signaling in controlling organ size, tumorigenesis as well as the insurgence of other important diseases in mammals. The ortholog human counterparts of core kinases Hpo and Warts are represented by the pro-apoptotic MST1/2 and LATS1/2 kinases [58, 59] (Figure 2). One ortholog exists for the adaptor protein Sav, termed WW45 or SAV1, and the other two orthologs for Mats are termed MOBKL1A and MOBKL1B (referred to as Mob1). These proteins form a conserved kinase cassette that phosphorylates and inactivates the mammalian Yki homologs YAP and TAZ [25, 47, 60] in response to cell density. This cell density-dependent activation of the Hippo pathway is required in contacting inhibition of cultured mammalian cells [47]. Similar to *Drosophila* Hippo signaling, all the mammalian components of the Hippo pathway clearly show tumor suppression activity. In fact, transgenic overexpression of YAP [61, 62] and liver-specific knockout of *Mst1/2* or *Sav1* [63–66] induce abnormal liver expansion in terms of size, and eventually hepatocellular carcinoma formation (HCC). YAP was initially identified as a 65 kDa binding partner of *c*-Yes from Sudol and collaborators [67]. YAP is a transcriptional co-activator of many transcription factors via its own WW-domain (reviewed in [68]). The TEAD/TEF family of transcription factors, whose homolog is represented by Sc in *Drosophila*, is considered the major partner of both YAP and TAZ in executing their activities within the Hippo pathway. The 4 mammalian TEF/TAED transcription factors are widely expressed and regulate transcription in specific tissues during certain development stages [69]. It was shown that TAED1/TEF2 and YAP share a large number of target genes [51, 70, 71]. In support of this evidence, TEAD1 and TEAD2 double-knockout mice display similar phenotypes to YAP knockouts [69]. Furthermore, ablation of TAED/TEF expression decreases the ability of YAP/TAZ in promoting anchorage independent growth and EMT (epithelial to mesenchymal transition) [51, 71, 72]. Recently Dupont and collaborators have identified YAP and TAZ as the nuclear principal complex of mechanical signals exerted by extracellular matrix (ECM) rigidity and cell shape. This regulation requires Rho GTPase activity and tension of the actomyosin cytoskeleton but is independent from the Hippo/LATS cascade. YAP/TAZ is required for differentiation of mesenchymal stem cells induced by ECM stiffness and for survival of endothelial cells regulated by cell geometry [73].

The exact role of YAP has yet to be defined since it appears to be able to act as an oncogene or as a tumor suppressor depending on the cellular context. YAP1 was shown to bind long forms of p73 and p63, while not to wt p53, thereby potentiating p73- and p63-induced apoptosis [74, 75]. In particular, p73 recapitulates the most well-characterized p53 antitumoral effects, from growth arrest and apoptosis to senescence. YAP imparts transcriptional target specificity to p73 in promoting either growth arrest or apoptosis in response to different stimuli [76–78].

3.2. The Complexity of Upstream Regulators: FRMD6, Mer, and Kibra. As mentioned above, the complexity of molecular links between the upstream regulators and the core kinases in mammals has not been clarified either for *Drosophila*. The mammalian genome contains homologs for all the reported upstream regulators of the Hippo pathway. Notably, it encodes more than one paralogue for each *Drosophila* component, thus increasing complexity and the need for further investigation. Two homologs for Kibra, KIBRA/WWC1 and WWC2 and for Expanded, FRMD6 and FRMD1, while only one for Merlin, NF2, were identified. Interestingly, they often differ in protein structure compared to *Drosophila* counterparts. One Ex homolog for FRMD6 does not possess the extended C-terminal portion that is required for growth inhibition activity of Ex and binding to Kibra [20, 79]. No interaction between FRM6 and MST1/2 has been confirmed, in contrast to the described interaction between Ex and Hippo [21]. Also Mer/NF2 is a FERM domain-containing protein and the most investigated. It is a tumor suppressor, whose mutations trigger neurofibromatosis 2, mainly characterized by tumor insurgence in the nervous system [80, 81]. It has a prominent role in growth inhibition triggered by C-adherin-based cell contact. Growth inhibitory action of Mer/NF2 appears to stem from controlling the distribution and signaling of membrane receptors. In fact, in Merlin K/D cells the activation and internalization of the EGF receptor are also maintained in high-cell-density conditions [82].

Furthermore, contrasting data for Mer/NF2 involvement in developing hepatocellular carcinoma (HCC) and tumors of the bile duct were reported. It is worthy to note that in specific *Merlin* $^{-/-}$ liver an increased proliferation of hepatocytes and of bile ducts was reported, coupled with minor LATS and YAP phopshorylation and increased YAP nuclear export [83]. Conversely, in this context, other authors did not observe any alterations in YAP phosphorylation and localization [84].

3.3. The Core Kinases: MST, LATS, and MOB. The ortholog human counterparts of core kinases Hpo and Warts are represented by the proapoptotic MST1/2 and LATS1/2 kinases [58, 59]. MST1/2 are serine-threonine kinases, better known for their ability to initiate apoptosis when overexpressed through a combination of p53- as well as JNK-mediated pathways [85, 86]. Generally, apoptosis induced by different stimuli is coupled with the activation of kinases MST1/2, which result themselves as substrates for caspases 3, 6, and 7 cleavage. This produces highly active catalytic fragments, which are mainly localized in the nucleus, where they exert their proapoptotic function [85–87]. As mentioned above, loss of function of the MST1/2 ortholog Hpo shows a phenotype characterized by a marked overgrowth due to accelerated cell-cycle progression and deregulated apoptosis. Exogenous MST2 expression can successfully rescue this phenotype. MSTs become activated by autophosphorylation in the threonine residues within their activation loop domain. Inhibition of dimerization and autophosphorylation of MST2 exerted by RAF1 was reported [88]. In this latter context, expression of *rassf1a* is able to release MST2 from RAF1 inhibition, thus inducing apoptosis [77]. Moreover, PP2A phosphatase dephosphorylates MST1/2 kinases as shown by two different groups [42, 89]. How autophosphorylation and activation of MST kinases are triggered by unknown extracellular stimuli remain to be elucidated, and okadaic acid treatment or siRNA-mediated knockdown of PP2A promote MST1/2 phopshorylation and activation. Interestingly, Guo and collaborators very recently showed that *rassf1a* activates MST1 and MST2 by preventing their dephosphorylation. Specifically, they observed that *rassf1a* knockdown, which is a frequent phenomenon in human tumors, leads to a dramatic decreased in MST1/2 levels exerted by phosphates. They also observed that restoring *rassf1a* expression and function promotes the formation of active MST1/2 by counteracting the role of phosphates. This is one of the first examples of a tumor suppressor acting as an inhibitor of a specific dephosphorylation pathway.

In the Hippo pathway context, MST substrates include LATS and MOB1. LATS1/2 kinases control cellular homeostasis, negatively regulating cell division cycle 2 (CDC2) and favoring G2/M arrest [90–92]. LATS2 was also reported to induce G1/S arrest [93]. In fact, both overexpressions of LATS1 and 2 dramatically inhibit both cell proliferation and anchorage-independent growth [47, 94] in various cell lines. It is also true that loss of LATS1/2 leads to a broad variety of tumors, such as soft tissue sarcoma and leukemia [95]. In light of these data, these proteins are believed to be strong tumor suppressors. Recent data addressed LATS involvement in tumor suppressive as well as oncogenic pathways, such as p53, RAS, and Akt signaling pathways. Interestingly, LATS2 can bind to MDM2 protein, thus inhibiting its E3 ubiquitin ligase activity to stabilize p53, which in turn favors the transcription of LATS2 [96]. Up until now, YAP and TAZ are the main LATS substrates identified in its kinase activity, but yet they only mediate some of the effects of LATS, thus indicating the existence of other substrates, such as Snail [97], DYRK1A [98], and LATS1 and LATS2 [99].

In the Hippo pathway context, LATS activity is supported by MOB1. This protein, which corresponds to the human ortholog of the Mats adaptor protein, binds to and phosphorylates LATS kinases, favoring YAP and TAZ protooncogenes phosphorylation and inhibiting their nuclear activity. MOB1 binding to LATS kinases is strongly enhanced upon phosphorylation of MOB1 by MST1/2 kinases [46]. Loss of MOB1 function results in increased cell proliferation and decreased cell death, suggesting that MOB1 functions, as well as the other Hippo pathway components, as a tumor suppressor protein.

4. *rassf1a* Signaling into Hippo Pathway

Due to the absence of enzyme activity, Ras-Association Domain Family (*rassf*) are noncatalytic-proteins. They are often referred to as "scaffold proteins," which are ubiquitously expressed in normal tissue and described in literature as a strong tumor suppressor family of proteins (reviewed in [100]). The *rassf*s family comprise ten members from *rassf1* to *rassf10*. Among them only *rassf1a* shares the closest homology to *Drosophila rassf* (*drassf*) (reviewed in [101]). *rassf1a* exhibits strong tumor suppressor function [102]. Loss of *rassf1a* allele is a frequent occurrence in primary human cancers [103, 104]. Furthermore, hypermethylation of *rassf1a* promoter is very often correlated with oncogenic phenotypes. Concomitantly, the identification of specific point mutations of *rassf1a* impinges on the ability of this protein to inhibit tumor cell growth [105, 106]. About 15% of primary tumors show point mutations of *rassf1a* [107]. Two independent research groups generated *rassf1a* knockout mice [108, 109]. Both these mice showed a phenotype with greatly increased susceptibility to tumor formation. Pursuing the hypothesis that the protein-protein interaction of YAP pattern changes as a consequence of different stimuli, Matallanas and colleagues followed the behavior of *rassf1a* after triggering apoptosis [77]. They showed that *rassf1a* disrupts the inhibitory complex between RAF1 and MST2 and favors the physical association between MST2 and LATS1 concomitantly, therefore, leading to YAP1 phosphorylation and nuclear relocalization where it binds to p73 and potentiates its apoptotic activity (Figure 2). It was also shown that the FAS active receptor induces *rassf1a* to compete with RAF1 in binding to MST2, thus promoting the formation of a LATS1 complex. This results in the translocation of YAP from the cytoplasm to the nucleus. These findings may suggest that the activation of the *rassf1a* complex indirectly diverts LATS1 from phosphorylating YAP, thus making it available for different phosphorylation events.

In addition, it is also able to enter into the nucleus where it can activate the transcription of p73 target genes involved in apoptosis.

It is worthy to note that in 2009, Hamilton and collaborators identified a novel DNA damage pathway that is activated by ATM kinase, involving *rassf1a* and Hippo pathway members [110]. They showed that, upon DNA damage, *rassf1a* becomes phopshorylated by ATM on Ser131. This event seems to be necessary in promoting MST2 binding to *rassf1a*, potentiating MST2 and LATS1 proapoptotic activity leading to p73 stabilization. Thus, this confirms findings observed in previous *in vitro* experiments showing that the *rassf1a* peptide containing an ATM putative domain is a substrate for ATM phosphorylation [111, 112].

More recently, the interaction, between *rassf1a* and SAV Hippo pathway member [113], was shown to potentiate p73-dependent apoptosis [114]. While this effect does not seem to require direct interaction between *rassf1a* and MST kinases, it was shown to trigger apoptosis via the MST/LATS pathway [77]. It is also true that SAV acts as a scaffold protein connecting MST kinases with LATS kinases [115] and that the expression of exogenous SAV can greatly enhance this proapoptotic signal [113]. Consequently, it is reasonable for authors to speculate the existence of a functional axis involving *rassf1a*-MST-SAV-LATS-YAP in promoting p73-induced apoptosis. Altogether, these findings show a close functional interconnection between *rassf1a*, Hippo, and p53 family tumor suppressor effects.

RASFF1A functions as a negative regulator of cardiomyocyte hypertrophy [116]. The latter displays an enlargement in size of cardiomyocytes, which is very often associated with heart failure [117]. It was proposed that a large number of protooncogenes, which are expressed in the heart, could possibly mediate this aberrant process [118]. *rassf1a* exon1*α* knockout mice exhibit normal cardiac morphology at 12 weeks of age. Notably, the application of a pressure overloaded the transverse aortic constriction causing massive cardiac hypertrophy, among the severest reactions ever to be reported [116]. This may suggest that *rassf1a* plays a role in contrasting overproliferation of cardiomyocytes. Interestingly, the authors observed that *rassf1a* in this cellular system greatly opposes the RAS-RAF1-ERK1/2 signal pathway. Not long ago, it was proposed that the activation of RAF by RAS requires a complex regulation of many adaptor molecules including the involvement of CNK1 (connector enhancer of kinase suppressor of RAS). This protein is able to form a complex with *rassf1a*, increasing *rassf1a*-induced cell death [119]. In light of these data authors speculated about a possible imbalance in the ratio of the components of the scaffold complex required for RAS signal transmission. CNK1 was also found to interact with MST1 and MST2, requiring MST kinases to induce apoptosis. Deleting the MST1 segment that mediates binding to *rassf1a* also eliminates the physical association between MST1 and CNK1. To sum up, CNK1 binds to *rassf1a* and promotes apoptosis through a pathway that requires *rassf1a* and MST kinases [119]. This mechanism may be the underlying factor behind *rassf1a*'s action in preventing cardiomyocytes hypertrophy. Supporting this, Del Re and collaborators showed that *rassf1a* is an endogenous activator of MST1 in the heart. They also found that in cardiac fibroblasts the *rassf1a*/MST1 pathway negatively regulates TNF-*α* that is believed to be a key mediator of hypertrophy and consecutive cardiac dysfunction [120]. Altogether, these findings highlight the importance of a crosstalk between *rassf1a* and components of the human Hippo pathway in preventing cardiac dysfunction due to aberrant overproliferation of cardiomyocytes. Of note, other Hippo pathway members were shown to be involved in heart development and size, such as YAP [121], Dch1-FAT [122], LATS2 [123], and SAV [124].

5. *rassf5* and *rassf6*

Other *rassf* family members were involved in modulating the activity of Hippo pathway components. The first RAS interactor discovered within this family was *rassf5* [125], often called Novel Ras Effector 1 (NORE1). This isoform that shares up to 60% homology with *rassf1*, is the most common isoform. As for many *rassf*s, it was demonstrated to be a centrosomal protein that can bind to the microtubule scaffold structure. This event appears to be required for growth inhibition and consequently tumor suppression activity, which is achieved through the inhibition of ERK signaling [126]. Furthermore, it has been reported that active RAS binds to *rassf5*-MST1 complex thereby conferring the role of the RAS effector complex in mediating the proapoptotic function of KiRASG12V [127]. RASFF5 and the MST1 pro-apoptotic kinase are involved in a physical interaction, thus forming an active complex where RAS interacts upon serum stimulation consequently leading to its pro-apoptotic function. Furthermore, the interaction of *rassf1a* and NORE1 with MST1 appears to be controversial. In fact, an inhibition of MST kinases activity by coexpression with the complex NORE1-*rassf1a* in excess was reported [128]. At the same time, by *in vivo* experiments, overexpression of *rassf1a* together with MST2 was shown to increase kinase activity of MST2 consequently potentiating its pro-apoptotic effect [77, 113, 129].

In 2009, Ikeda and collaborators showed that another *rassf* member, *rassf*6, can bind to MST2 kinase. This protein is known to induce apoptosis [130, 131]. When *rassf*6 is bound to MST2, *rassf*6 inhibits MST2 activity, thus, inhibiting its role in the Hippo pathway. Conversely, the release of MST2 from *rassf*6 causes apoptosis in a WW45-dependent manner (*Drosophila* SAV). Therefore, *rassf*6 impinges the Hippo proapoptotic pathway by inhibiting MST2, but it is *per se* able to induce apoptosis through a parallel Hippo mechanism. In fact, MST2 is responsible for apoptosis induced through Hippo signaling and through a *rassf6*-WW45-mediated pathway [131].

6. Concluding Remarks and Future Perspectives

In conclusion, the Hippo pathway is a signaling pathway that regulates cell proliferation and cell death. It is a kinase cascade that phosphorylates and negatively regulates transcription by transcriptional coactivators. As summarized

above, the loss of function of the Hippo pathway triggers tumorigenesis. Accordingly, the downregulation of the Hippo pathway is frequently observed in human cancers. Aberrant activation of Hippo downstream executors, YAP1 and TAZ, induce epithelial-mesenchymal transition and the expression of stem-cell markers in cancer cells. Quite recently, the Hippo and the *rassf* pathways have emerged to be closely linked. The tumor suppressor *rassf* proteins were shown to induce cell-cycle arrest and apoptosis. Stimuli activating the Hippo pathway simultaneously induce *rassf*-dependent biological events. Thereby, the Hippo and *rassf* pathways cooperate in preventing tumorigenesis. Reintegration of the Hippo pathway and *rassf* functions should be implemented in cancer therapy. However, it is also true that if this cross-talk results disproportionate, the consequence will be excessive apoptosis and consecutive organ dysfunction. In such cases, the involvement of the Hippo/*rassf* inhibitors will be useful. The relationship between the Hippo and *rassf* pathways is probably not restricted to cancer biology since many of the Hippo components also regulate adipogenesis, osteogenesis, and myogenesis. As discussed above, a growing body of evidence shows that this relationship between *rassf* and the Hippo pathways also occurs in cardiac tissue inhibiting cardiac hypertrophy and playing a critical role in preventing heart failure. Based on what has been described and in light of the synergistic effects observed on the interaction within *rassf* and components of Hippo signaling in preventing defects of proper biological development such as insurgence of many human diseases, much more work is needed to further investigate the importance of this physiological relationship.

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Review Article

Modulator of Apoptosis 1: A Highly Regulated RASSF1A-Interacting BH3-Like Protein

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Modulator of apoptosis 1 (MOAP-1) is a BH3-like protein that plays key roles in both the intrinsic and extrinsic modes of cell death or apoptosis. MOAP-1 is part of the Ras association domain family 1A (RASSF1A)/MOAP-1 pro-apoptotic extrinsic signaling pathway that regulates apoptosis by utilizing death receptors such as tumor necrosis factor α (TNFα) or TNF-related apoptosis-inducing ligand (TRAIL) to inhibit abnormal growth. RASSF1A is a bona fide tumor suppressor gene that is epigenetically silenced by promoter-specific methylation in numerous human cancers. MOAP-1 is a downstream effector of RASSF1A that promotes Bax activation and cell death and is highly regulated during apoptosis. We speculate that MOAP-1 and RASSF1A are important elements of an "apoptotic checkpoint" that directly influences the outcome of cell death. The failure to regulate this pro-apoptotic pathway may result in the appearance of cancer and possibly other disorders. Although loss of RASSF1A expression is frequently observed in human cancers, it is currently unknown if MOAP-1 expression may also be affected during carcinogenesis to result in uncontrolled malignant growth. In this article, we will summarize what is known about the biological role(s) of MOAP-1 and how it functions as a downstream effector to RASSF1A.

1. Introduction

Cancer is a disease of uncontrolled cell proliferation and is the third leading causing of death worldwide following cardiovascular and infectious diseases [2]. The abnormal proliferation of cells during cancer development results from a multistep process involving the deregulation of genes that promote cell growth (oncogenes) and those that normally function to restrain growth (tumor suppressors). Interestingly, approximately 90% of the genes that are associated with cancer development have now been identified as being tumor suppressors [3]. Moreover, many of these growth inhibitory genes encode proteins that are involved in cell death. RASSF1A has multiple biological functions including the regulation of Bax-mediated cell death [4–6]. MOAP-1, a highly regulated pro-apoptotic protein, serves a critical role during mitochondrial-dependent apoptosis by influencing and sustaining Bax activation [7, 8]. In this review, we will discuss how MOAP-1 is regulated and how it serves as a pivotal RASSF1A effector protein to regulate cell death.

2. Apoptosis: A Regulated Biological Process to Modulate Growth

A well-known mechanism of tumor suppression is the elimination of unwanted cells through a sequence of events known as *apoptosis* [9]. The significance of apoptosis in metazoan biology is highlighted by the number of diseases that are associated with its deregulation [10]. Apoptosis plays a critical role during the development of multicellular organisms and adult tissue homeostasis and is vital to the removal of damaged or dangerous cells. It can be initiated through two main pathways in response to intracellular or extracellular signals of cell death [11]. The intrinsic apoptotic signaling pathway is activated in response to a diverse set of signals originating from within cells due to cellular stresses such as DNA damage, hypoxia, toxins, or starvation [12]. In contrast, the extrinsic pathway of cell death is activated by the binding of death-inducing ligands to death receptors.

Activation of the extrinsic apoptotic signaling pathway occurs through cell surface death receptor/ligand

combinations that include TNF-R1/TNFα, Fas receptor (R) (CD95/ APO-1)/Fas ligand, as well as TRAIL-R (1/2)/TRAIL [13]. Activated death receptors trigger a series of events resulting in the formation of trimeric receptor complexes and the death-inducing signaling complex (DISC) [14]. DISC assembly and subsequent activation of initiator caspases (mainly caspase-8) convey signals to the mitochondria to promote the release of small molecules (such as cytochrome c) from the mitochondrial matrix into the cytosol and the assembly of the apoptosome complex to activate downstream effector caspases (such as caspase-3) [15]. Intrinsic pathway stimulation can also lead to cytochrome c release and activation of effector caspases. Once activated, effector caspases cleave several nuclear proteins [such as lamin B and poly(ADP-ribose) polymerase] and activate specific DNA endonucleases. These events result in many of the biochemical and morphological changes observed during apoptosis, including nuclear and cytoplasmic breakdown.

Mitochondria play an important role in the induction of apoptosis through the release of proteins that promote caspase activation and the breakdown of cellular components [16]. Regulation of the mitochondrial events during apoptosis is controlled by proteins of the B-cell lymphoma-2 (Bcl-2) family and is composed of three different subgroups known as the anti-apoptotic, multidomain pro-apoptotic and BH3-only proteins [12, 17]. The anti-apoptotic and BH3-only proteins are involved in inhibiting or promoting the function of multi-domain pro-apoptotic molecules, respectively. In contrast, it is members of the multidomain subgroup that are directly responsible for the mitochondrial outer membrane permeabilization that occurs during apoptosis [18, 19]. Two members from this group, Bax and Bak, are required for apoptosis to occur [20]. Although the functions of Bax and Bak are closely regulated by its Bcl-2 family members, it is now known that, for at least Bax activation, other proteins may also be involved in its modulation. One of these molecules is the RASSF1A-binding protein, MOAP-1. RASSF1A functions to "open" MOAP-1 to allow for MOAP-1-induced Bax conformational change by exposing the epitope, ^{12}GPTSSEQIMKTGA^{24}, and allowing for the subsequent insertion of Bax into the mitochondrial membrane. Once inserted, Bax can cooperatively drive cell death in association with Bak [21].

3. Ras Association Domain Family

RASSF1A is a bona fide tumor suppressor molecule that serves as the founding member of the RASSF group of proteins [22]. Currently, the RASSF protein family is comprised of ten different members known as RASSF1–10 that each share the presence of a Ras association (RA) domain within its primary amino acid sequence [23–26]. Of this protein family, RASSF1 is the most thoroughly characterized and studied thus far. A loss or decrease in RASSF1A expression is frequently observed in a wide range of human cancers due to epigenetic transcriptional silencing [27–30].

The tumor suppressor functions of RASSF1A include the ability to regulate microtubule dynamics [31–33], mitosis [32, 34–37], and apoptosis [5, 6, 38–41]. Due to the particular focus of this paper, we will only discuss in detail what is known about RASSF1A-dependent cell death involving MOAP-1. It is now known that several pro-apoptotic pathways can be modulated by RASSF1A. One such pathway for the induction of RASSF1A-mediated apoptosis involves protein interactions with the Hippo signaling components, serine/threonine kinases mammalian Ste20-like (MST) 1 and 2 (reviewed separately in this issue). The Hippo pathway is a conserved signaling pathway essential for organ growth regulation in *Drosophila* and vertebrates [42]. Currently, there is evidence to support the role for RASSF1A in modulating the kinase activity of MST1/2 and thus MST1/2-mediated cell death [38, 39]. RASSF1A can also induce apoptosis through an MST2-specific pathway by releasing MST2 from its inhibitor, Raf1, and allowing for large tumor suppressor homology (*Drosophila*) (LATS)1-mediated activation of the transcriptional regulator Yes-associated protein (YAP)1 [41]. In turn, YAP1 can translocate to the nucleus and associate with the p73 transcription factor in order to induce the transcription of pro-apoptotic gene p53-upregulated modulator of apoptosis (*PUMA*) to aid in Hippo-mediated cell death.

A second pathway involves MOAP-1. In response to death receptor signaling involving TNFα or TRAIL, RASSF1A can associate with MOAP-1 in order to promote Bax conformational change, translocation and integration into the mitochondrial membrane to perturb mitochondrial permeability [5, 6]. This is followed by the release of cytochrome c to activate downstream caspases and to promote nuclear and cytoplasmic breakdown. Furthermore, we speculate that MOAP-1 may cooperate with RASSF1A to promote tumor suppression. RASSF1A has been extensively reviewed in the literature. In contrast, there are currently no reviews that specifically address what is known about the biology of MOAP-1. Indeed, MOAP-1 remains separate from the canonical group of Bax-regulatory molecules and therefore has not garnered as much attention as the proteins of the Bcl-2 family. In the remainder of this review, we will document what is currently known about MOAP-1 and will discuss evidence providing insight into the complexities of this protein and its biological function(s).

4. Modulator of Apoptosis 1: A Brief History

MOAP-1 was first reported as a mitochondria-enriched 39.5 kDa molecule that was first identified as a novel Bax-associating protein in a yeast two-hybrid screen [7]. Located at genetic locus 14q32 (Figure 1), MOAP-1 is a negatively charged protein that contains 351 amino acid residues in humans and an isoelectric point (pI) of 4.939 at pH 7.0 (Ensembl protein ID: ENST00000298894). *MOAP-1* is highly conserved in chimpanzee (*Pan troglodytes*), rat (*Rattus norvegicus*) and mouse (*Mus musculus*), and its coding sequence is contained within a single exon in both mouse and humans (Figure 2). Since its discovery in 2001, research has established a central role for MOAP-1 in both mitochondrial and death receptor-mediated apoptosis [5, 8]. When overexpressed in mammalian cells, MOAP-1 induces caspase-dependent apoptosis whereas MOAP-1

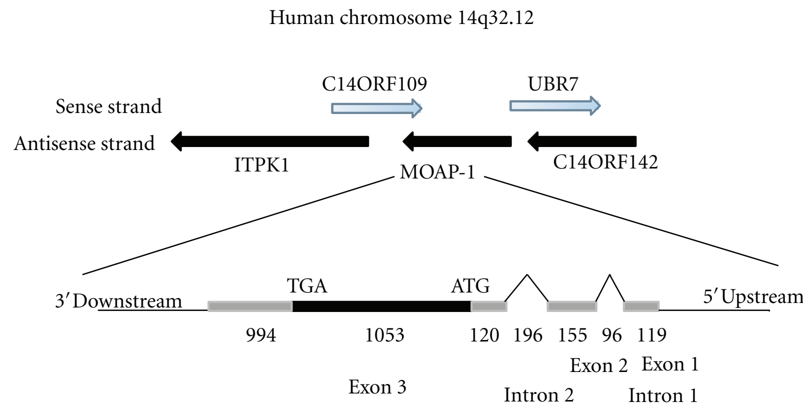

Figure 1: Gene structure of human MOAP-1. The entire protein coding sequence of MOAP-1 is contained within exon 3 and is located on the anti-sense strand of chromosome 14. Genbank accession: NM_022151.4. More information can be found at http://www.ncbi.nlm.nih.gov/gene/64112. Numbers below schematic denote the size of the intron or exon.

knockdown cells are resistant to a variety of apoptotic stimuli including staurosporine, serum withdrawal, UV irradiation, TNFα, and TRAIL [8]. Altogether, these results demonstrate the importance of MOAP-1 in apoptosis and functions as a key effector of Bax conformational change and activation.

5. MOAP-1 Expression in Normal and Cancer Cells

MOAP-1 is a ubiquitously expressed protein that is present at moderate levels under normal cellular conditions and is constitutively degraded by the ubiquitin-proteasome system [7, 43]. Given that RASSF1A expression is frequently lost during carcinogenesis and Bax is mutated in a large percentage of gastrointestinal and colorectal cancers, it is plausible that MOAP-1 expression and/or function may also be regulated during cancer development [29, 44, 45]. Indeed, immunohistochemical analysis of MOAP-1 performed over a wide range of human cancer tissues demonstrates either a negative or a weak staining pattern for this protein (Table 1 and please see site http://www.proteinatlas.org/search/moap1 under "moap1 or pnma4" for immunohistochemical pictures of MOAP-1 staining in numerous cancer cells). In support of this immunohistochemical data, we have also found a loss or reduction of MOAP-1 expression in an extensive panel of cancer cell lines ranging from breast, brain, lung, skin and blood cancers [Law et al., unpublished observations]. Furthermore, in a classical xenograft assay, both RASSF1A and MOAP-1 can suppress tumor formation in HCT116 colon cancer cells suggesting tumor suppressor function (Figure 3) and functional importance for both genes in growth inhibition in normal cells.

Table 1: Summary of MOAP-1 staining patterns in human malignant tissues. Data source was The Human Protein Atlas (http://www.proteinatlas.org/search/moap1). Antibody used for all MOAP-1 immunohistochemistry: Sigma-Aldrich HPA000939.

Cancer tissue type	MOAP-1 staining pattern
Colorectal cancer	Weak
Breast cancer	Negative
Prostate cancer	Negative
Ovarian cancer	Negative
Cervical cancer	Negative
Endometrial cancer	Negative
Malignant carcinoid	Negative
Head and neck cancer	Negative
Thyroid cancer	Negative
Malignant glioma	Weak
Malignant lymphoma	Negative
Lung cancer	Weak
Malignant melanoma	Negative
Skin cancer	Negative
Testis cancer	Moderate
Urothelial cancer	Negative
Renal cancer	Negative
Stomach cancer	Weak
Pancreatic cancer	Negative
Liver cancer	Negative

Currently, the mechanism responsible for the loss of MOAP-1 expression in cancer cells remains unknown. It is possible that expression changes in MOAP-1 may arise by promoter specific epigenetic methylation, by miRNA/siRNA regulation of the mRNA, and/or by alterations in MOAP-1 protein stability due to ubiquitin-directed proteolysis. The *MOAP-1* promoter displays 17 potential CpG islands that may be epigenetically modified to result in loss of gene expression, as suggested using MethPrimer online software [46]. To date, no miRNA or siRNA has been identified for MOAP-1 although we suspect that specific miRNA(s) may exist to reduce or shut down MOAP-1 expression. The last potential mechanism regulating MOAP-1 expression is posttranslational modification by ubiquitination and degradation by the proteasomal degradation machinery [43]. Future investigations will be required in order to understand

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hMOAP-1  MTLRLLEDWCRGMDMNPRKALLIAGISQSCSVAEIEEALQAGLAPLGEYRLLGRMFRRDE  60
cMOAP-1  MTLRLLEDWCRGMDMNPRKALLIAGISQSCSVAEIEEALQAGLAPLGEYRLLGRMFRRDE  60
mMOAP-1  MTLRLLEDWCRGMDMNPRKALLVAGIPPTCGVADIEEALQAGLAPLGEHRLLGRMFRRDE  60
rMOAP-1  MTLRLLEDWCRGMDMNPRKALLVAGIPPTCGVADIEEALQVGLAPLGEHRLLGRMFRRDE  60
         **********************:***. :*.**:******.*******:***********

hMOAP-1  NRKVALVGLTAETSHALVPKEIPGKGGIWRVIFKPPDPDNTFLSRLNEFLAGEGMTVGEL  120
cMOAP-1  NRKVALVGLTAETSHALVPKEIPGKGGIWRVIFKPPDPDNTFLSRLNEFLAGEGMTVGEL  120
mMOAP-1  NKNVALIGLTVETGSALVPKEIPAKGGVWRVIFKPPDTDSDFLCRLNEFLKGEGMTMGEL  120
rMOAP-1  NKNVALVGLTVETGSALVPKEIPAKGGVWRVIFKPPDADSDFLCRLNEFLKGEGMTMGEL  120
         *::***:***.**. ********.***:*********.*. **.****** *****:***

hMOAP-1  SRALGHENGSLDPEQG-MIPEMWAPMLAQAL-EALQPALQCLKYKKLRVFSGRESPEPGE  178
cMOAP-1  TRALGHENGSLDPEQG-MVPEMWAPMLAQAL-EALQPALQCLKYKKLRVFSGRESPEPGE  178
mMOAP-1  TRVLGNRNDPLGLDPGIMIPEIRAPMLAQALNEALKPTLQYLRYKKLSVFSGRDPPGPGE  180
rMOAP-1  TRVLGNRNDPLCLDQNVMIPEIRAPMLAQALDEALKPTLQYLRYKKLSVFSGRDPPGPGE  180
         :*.**:.*..*  : . *:**: ******** ***:*:** *:**** *****:.* ***

hMOAP-1  EEFGRWMFHTTQMIKAWQVPDVEKRRRLLESLRGPALDVIRVLKINNPLITVDECLQALE  238
cMOAP-1  EEFGRWMFHTTQMIKAWQVPDVEKRRRLLESLRGPALDVIRVLKINNPLITVDECLQALE  238
mMOAP-1  EEFESWMFHTSQVMKTWQVSDVEKRRRLIESLRGPAFEIIRVLKINNPFITVAECLKTLE  240
rMOAP-1  EEFESWMFHTSQVMKTWQVSDVEKRRRLMESLRGPAFEIIRVLKINNPFITVAECLKTLE  240
         ***  *****:*::*:***.*******:*******:::*********:*** ***::**

hMOAP-1  EVFGVTDNPRELQVKYLTTYQKDEEKLSAYVLRLEPLLQKLVQRGAIERDAVNQARLDQV  298
cMOAP-1  EVFGVTDNPRELQVKYLTTYQKDEEKLSAYVLRLEPLLQKLVQRGAIERDAVNQARLDQV  298
mMOAP-1  TIFGIIDNPRALQVKYLTTYQKTDEKLSAYVLRLEPLLQKLVQKGAIEKEVVNQARLDQV  300
rMOAP-1  TIFGIIDNPRALQVRYLTTYQKSGEKLSAYVLRLEPLLQKLVQKGAIEKEVVNQARLDQV  300
          :**: **** ***:*******  ******************:****::.*********

hMOAP-1  IAGAVHKTIRRELNLPEDGPAPGFLQLLVLIKDYEAAEEEEALLQAILEGNFT  351
cMOAP-1  IAGAVHKTIRRELNLPEDGPAPGFLQLLVLIKDYEAAEEEEALLQAVLEGNFT  351
mMOAP-1  IAGAVHKSVRRELGLPEGSPAPGLLQLLTLIKDKEAAEEE-VLLQAELEGYCT  352
rMOAP-1  IAGAIHKSVRRELGLPEGSPAPGLLQLLTLIKDKEAAEEE-VLLQAELEGHFT  352
         ****:**::****.***..****:****.**** ** *** .**** ***  *
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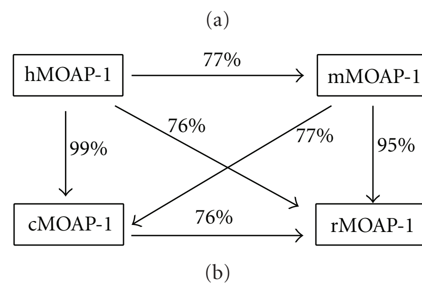
(a)



(b)

FIGURE 2: A comparison of MOAP-1 orthologs. (a) Multiple sequence alignments of MOAP-1 orthologs present in human (h), mouse (m), rat (r) and chimpanzee (c). Sequence alignments were performed using ClustalW2. NCBI reference sequences (mRNA and protein): NM_022151.4 and NP_071434.2 (human); NM_022323.7 and NP_071718.1 (mouse); NM_001013101.1 and NP_001013119.1 (rat); XM_510137.3 and XP_510137 (chimpanzee). (b) Percent amino acid identity between MOAP-1 orthologs calculated based on sequence alignments in (2a). Analysis carried out using ClustalW2.

the ubiquitination of MOAP-1 and the biological outcome of these ubiquitination events.

Like most disease-associated genes, polymorphisms may exist to result in the loss of the encoded protein function. Single nucleotide polymorphisms (SNPs) of MOAP-1 have been documented in two databases suggesting disease-associated changes [47, 48]. Although the population distribution has not been determined as of yet, two somatically

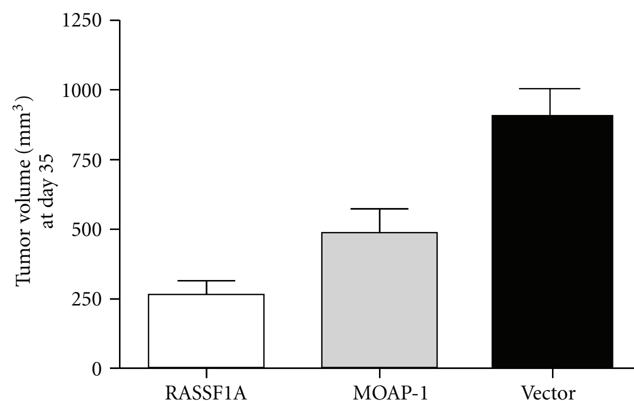


Figure 3: Tumor inhibiting potential of the RASSF1A/MOAP-1 tumor suppressor pathway. A classical xenograft assay was carried out. Male athymic nude mice were injected subcutaneously with 1×10^6 transiently transfected HCT116 cells mixed with matrigel mix into the right and left flank areas. Tumor volumes were measured until day 35 and plotted. *P* values for MOAP-1 versus vector (0.019); RASSF1A versus vector (0.0001); MOAP-1 versus RASSF1A (0.02), n = 12–14. Statistical analysis was evaluated by Student's *t*-test (two-tailed). Protein expression at the time of subcutaneous injection was confirmed by immunoblotting (data not shown). Protein expression in HCT116 cells can be detected up to 10 days post-transfection. However, at the end of experiment, we could not detect protein expression of HA-RASSF1A or Myc-MOAP-1 in the resulting tumors. We argue that the growth properties of HCT116 cells containing the indicated expression constructs were programmed within the first 7–10 days and continued on that program even though expression detection of the indicated genes was not possible. Please refer to [1] for more details on this issue.

derived SNPs (resulting in a predicted amino acid change) have been observed in melanoma patients—a proline to serine change at amino acid 79 (P79S with a nucleotide change of CCT → TCT) and an alanine to aspartic acid change at position 335 (A335D with a nucleotide change of GCT → GAT) [49]. Interestingly, the P79S polymorphism may suggest the creation of a potentially novel serine phosphorylation site to affect the cell death properties of MOAP-1, whereas A335D amino acid change would affect the TNF-R1-binding site on MOAP-1 (please see Figure 4). Further verification of these SNPs is warranted with respect to penetrance within the normal and disease groups, origin of these potential polymorphic changes, and their biological significance. Regardless of how MOAP-1 may lose expression and/or function, we speculate that the combined loss of both MOAP-1 and RASSF1A expression may be a common event occurring during carcinogenesis to result in the functional loss of the MOAP-1/RASSF1A cell death pathway and enhanced proliferation of malignant cells. Furthermore, the absence of MOAP-1 in cancer cells would also impact to some extent on the intrinsic apoptotic pathway(s) where MOAP-1 has been shown to play a role [8] and which is also the target of many chemotherapeutic drugs. Future investigations will be required in order to determine the cause(s) underlying MOAP-1 expression changes in human cancer.

Evidence from the literature indicates downregulation of *MOAP-1* expression in macrophage cells upon overexpression of the transcription factor MafB [50]. Upregulation of MafB is commonly observed in alveolar macrophages that have been exposed to cigarette smoke, and, incidentally, these cells also display increased viability [50, 51]. It has been proposed that MafB may promote macrophage survival through inhibition of apoptosis, which may be achieved through downregulation of pro-apoptotic molecules such as MOAP-1 [50]. In addition, analysis of the promoter region of MOAP-1 for transcription factor binding sites identified several interesting sites for NFκB (CCCTGGTCCC CAAGGAAATA CCT GCAAAAG) and c-Rel (ATCGGAATGA CCCTCTCGGC) and three sites for STAT1 (CTTGCTCCCT TAGGGGAACA) using the online, publicly available Transcription Factor Search (TFSEARCH) software. It remains to be determined if these are functional transcription factor binding sites but does provide hints to the complexity of MOAP-1 expression and reaffirms its importance in both cell death and growth control.

6. Interaction of MOAP-1 with Bcl-2 Family Members

As a pro-apoptotic molecule, MOAP-1 selectively interacts with members of the Bcl-2 protein family. In particular, its association with Bax requires the presence of a Bcl-2 homology 3 (BH3)-like domain within amino acids 120–127 and the same domain is also essential for mediating apoptosis [7]. Interestingly, the association of MOAP-1 to Bax requires all three BH (BH1, BH2, BH3) domains of the latter protein and is thus in contrast to other known Bax-associating partners (Figure 4). Additionally, it is speculated that MOAP-1 may associate at the hydrophobic cleft of Bax since critical point mutations in any of the three BH domains in Bax result in a loss of MOAP-1 association. The interaction between MOAP-1 and Bax occurs upon induction of apoptosis in response to activators of both the intrinsic and extrinsic cell death pathways and facilitates the release of cytochrome c from the mitochondria [8].

In addition to Bax, MOAP-1 also associates with the pro-survival anti-apoptotic proteins Bcl-2 and Bcl-X_L but not additional Bcl-2 family members Bid, BimL, Bak, Bad or Bcl-w under the same experimental conditions [7]. Evidence suggests that its interactions with Bcl-2 and Bcl-X_L may function to restrain the pro-apoptotic activity of MOAP-1 since overexpression of Bcl-X_L is sufficient to block MOAP-1-mediated cell death. Therefore, it appears that MOAP-1 may function similar to the canonical BH3-only proteins of the Bcl-2 family that are known to promote Bax activation and which are also inhibited by its anti-apoptotic family members.

7. Cooperation of MOAP-1 with RASSF1A in Death Receptor-Mediated Apoptosis

MOAP-1 is required for execution of both the intrinsic and extrinsic pathways of apoptosis where it is required for Bax

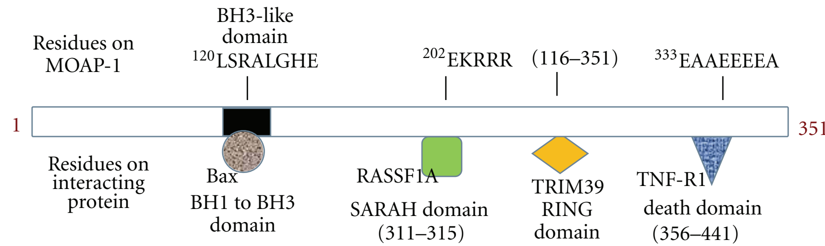


Figure 4: A schematic of MOAP-1 with indicated areas of contact with other proteins documented above schematic. Residues empirically determined to be required for protein interactions are indicated below each region.

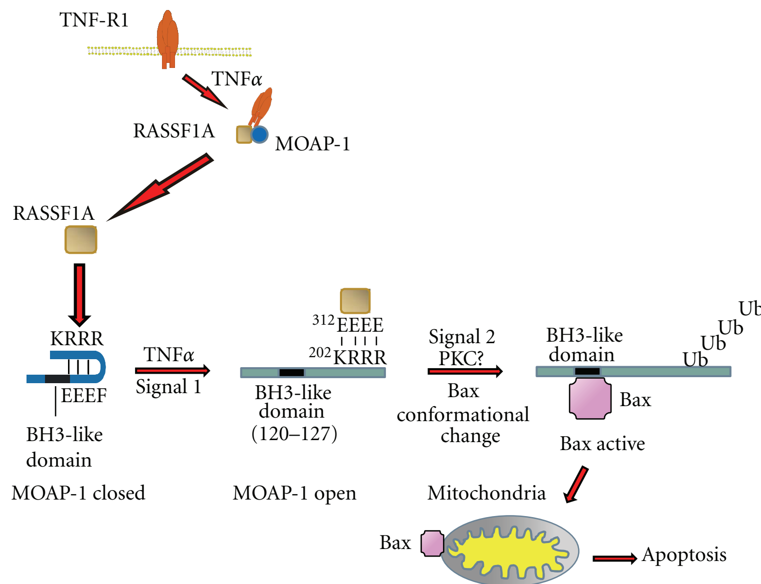


Figure 5: MOAP-1 cooperates with RASSF1A during death receptor-dependent apoptosis and promotes Bax activation. In response to death receptor stimulation, MOAP-1 is first recruited to the receptor and then followed by RASSF1A association at the MOAP-1/receptor complex. The association of MOAP-1 to RASSF1A promotes a conformational change in MOAP-1 that exposes its BH3-like domain required for Bax association. The subsequent interaction between MOAP-1 and Bax induces a conformational change in Bax that enables its translocation, and insertion into the mitochondrial outer membrane resulting in the release of cytochrome c and other apoptogenic factors, leading to apoptosis.

conformational change and translocation from the cytosol to the mitochondria prior to the release of apoptogenic factors [5, 8]. Although the mechanistic details of its role in the intrinsic pathway are currently unknown, the death receptor-dependent pathway involving MOAP-1 has been delineated to a great extent [5, 6] (Figure 5).

Under nonstimulated conditions, MOAP-1 is normally held in a “closed” conformation through an intraelectrostatic interaction involving regions ^{178}EEEF and ^{202}KRRR [6]. However, stimulation of cells with TNFα or TRAIL results in the recruitment of MOAP-1 to the receptor via a basic sequence (^{336}EEEEA) at its C-terminal end (Figures 4 and 5). Prior to death receptor association, RASSF1A is released from association with 14-3-3 and loses its ability to homodimerize [52]. Upon binding to the receptor through its N-terminal cysteine-rich (C1) domain, RASSF1A induces a conformational change in MOAP-1 to a more “open” state (Figure 5, Signal 1, TNFα) that exposes its BH3-like domain and allows it to bind and promote the activation of Bax [6].

The association of MOAP-1 with RASSF1A involves the sequence ^{202}KRRR in the former protein and ^{312}EEEE in the latter. Although activated K-Ras has been reported to be required for stabilization of the MOAP-1/RASSF1A protein complex [53], we are is able to consistently detect robust associations between MOAP-1 and RASSF1A in experiments that do not require the presence of overexpressed active K-Ras [5, 6]. Therefore, we are currently unable to explain or support the results of Vos and colleagues. Nonetheless, MOAP-1-induced Bax conformational change enables Bax to translocate from the cytosol to the mitochondria where it can insert into the mitochondrial membrane and promote the release of cytochrome c as well as other apoptosis-inducing factors, resulting in cell death. Therefore, MOAP-1 functions alongside RASSF1A as a key component linking

death receptor signaling to Bax activation and mitochondria-associated cell death. The MOAP-1/RASSF1A pathway exists as a separate, parallel signaling cascade that links the extrinsic and intrinsic pathways of apoptosis independent of tBid and caspase 8 [5].

In addition to RASSF1A, MOAP-1 has also demonstrated the ability to associate with a second RASSF family member, RASSF6 [54]. The interaction between RASSF6 and MOAP-1 is enhanced by the presence of activated K-Ras, and, furthermore, RASSF6 is also able to promote apoptosis. Therefore, it has been speculated that Ras may activate the pro-apoptotic function of RASSF6 and that RASSF6 may cooperate with MOAP-1 in a pathway similar to RASSF1A in order to induce cell death. However, this hypothesis still needs to be verified.

8. Regulation of MOAP-1 Stability by Apoptotic Signals

Under nonstimulated conditions, MOAP-1 is constitutively degraded by the ubiquitin-proteasome system and is normally a short-lived protein with a half-life of approximately 25 minutes [43]. However, evidence suggests that targeting of MOAP-1 to the proteasome may involve an unconventional mechanism given that no specific lysine residue can be identified as the site of polyubiquitination [43]. Indeed, mutation of any individual lysine residue or combination of residues fails to abolish MOAP-1 ubiquitination. Thus, the process involved in controlling MOAP-1 turnover remains to be determined.

In addition to regulation of basal MOAP-1 expression, MOAP-1 is also rapidly upregulated in response to multiple apoptotic stimuli including serum withdrawal, etoposide, TRAIL, and the endoplasmic reticulum stress inducer thapsigargin [43]. The increase in MOAP-1 protein arises through inhibition of its polyubiquitination and subsequent proteasomal degradation. Research findings demonstrate that elevation of MOAP-1 levels occurs prior to cell commitment to apoptosis and that the stabilization of MOAP-1 helps to sensitize cells to apoptosis by increasing the levels of activated Bax.

Intriguingly, stabilization of MOAP-1 in response to apoptosis employs the RING domain protein tripartite motif containing 39 (TRIM39) [55]. TRIM39 has not yet been functionally characterized but belongs to the tripartite motif (TRIM) family of proteins that are commonly involved in innate immunity [56] and contains three zinc-binding domains including a RING, B box, and coiled-coil region. Although a large number of proteins that contain RING domains also function as E3 ligases [57], TRIM39 associates with MOAP-1 in a manner that promotes its stabilization rather than its polyubiquitination [55]. TRIM39 also sensitizes cells to apoptosis by inhibiting MOAP-1 ubiquitination (through an unknown mechanism) and thus allows for the accumulation of MOAP-1 that can then can activate Bax. Furthermore, it was observed that both TRIM39 and MOAP-1 influence each other's localization to the mitochondria when overexpressed in HEK293 cells [55]. The upregulation of MOAP-1 protein levels can also occur in response to chemical toxins and clinical drugs reaffirming our speculation that MOAP-1 in cancer cells may be important for patient response to certain chemotherapeutic treatments [58]. Incubation of chronic lymphocytic leukemia cells with the apoptosis-inducing compound 5-aminoimidazole-4-carboxamideriboside or acadesine (AICAR) has been shown to result in a significant increase in *MOAP-1* expression [58]. Although the pathway through which AICAR induces cell death remains unknown, it is achieved through a mechanism that is independent of both AMPK and p53. In a second example, the addition of the novel immunosuppressant 2-amino-2[2-(4-octylphenyl) ethyl]-1,3-propane-diol hydrochloride (FTY720) to Jurkat cells results in a greater than tenfold upregulation of MOAP-1 mRNA levels [59]. It is believed that the potent immunosuppressive function of FTY720 may be attributed to its ability to induce lymphocyte apoptosis [60]. However, FTY720 has also been shown to induce apoptosis in a variety of different cancer cell types and to prevent breast cancer metastasis in mouse models [61–64]. Thus, it is plausible that the immunosuppressive and/or antitumorigenic effects of FTY720 may be partially mediated by MOAP-1.

We have evidence for a nondegradative ubiquitination of MOAP-1. This post-translational modification proceeds through a mechanism that is responsive to death receptor stimulation and a novel protein kinase C (PKC) dependent event [Law et al, unpublished observations] that may allow MOAP-1 to associate with and promote Bax activation (Figure 5, Signal 2). Interestingly, MOAP-1 has two potential binding sites for TRAF2, an E3-ubiquitin ligase important for TNF-R1-dependent signaling. These sites are at ^{178}EPGEEFGRW AND ^{331}DYEAAEEEAL with the underlined residues forming the core of the TRAF2 association site [64]. The first potential site is part of the intraelectrostatic pair that overlaps with the BH3-domain of MOAP-1. We are currently investigating the possible involvement of TRAF2 in MOAP-1 ubiquitination and the functional importance of several potential lysine residues for ubiquitin-dependent modification. We speculate that the ubiquitination of MOAP-1 may influence MOAP-1-mediated growth suppression and/or MOAP-1-directed apoptosis. This form of MOAP-1 ubiquitination adds to the complexity of MOAP-1 stability by a degradative-dependent ubiquitination to modulate the biological functions of MOAP-1.

9. MOAP-1: A Paraneoplastic Antigen

In addition to its role as a pro-apoptotic molecule, MOAP-1 is also the fourth member of the paraneoplastic Ma antigen (PNMA) family and is consequently also known as PNMA4. Paraneoplastic antigens (also termed "onconeural antigens") are proteins that are restricted in expression to immune-privileged sites within the body (such as the brain) and are therefore recognized as foreign molecules by the immune system when aberrantly expressed at other sites [65, 66]. Remarkably, these foreign proteins are expressed by systemic tumors in a subset of cancer patients which subsequently trigger an immune-mediated antitumor response. In some patients, this immune response is not only directed against

the tumor itself but also towards the sites within the body that ordinarily express the protein. In the case of the brain, this immune response results in neuronal degeneration and the development of an autoimmune neurologic disease known as a paraneoplastic neurological disorder (PND).

The PNMA family consists of six members (PNMAs 1–6) that, with the exceptions of PNMAs 4, 5, and 6, were originally identified through screening of complementary DNA libraries using antibody-containing sera from patients with PNDs [67]. Although MOAP-1/PNMA4 is ubiquitously expressed with higher levels in the heart and brain [7], each of the other family members are more restricted in expression [67–71]. The detection of antibodies to PNMAs 1–3 in PND patients is associated with disorders affecting the limbic system, brain, stem and cerebellum but is not indicative of any particular cancer type [68–70, 72]. In contrast, MOAP-1 has a well-established role in apoptosis and—similar to PNMA5 and PNMA6—is not associated with the development of PNDs to date. MOAP-1 displays the greatest amino acid sequence homology with PNMA1 (58%) which functions as a neuronal-specific pro-apoptotic molecule [73]. PNMA1 contains both a BH3-like domain and a conserved RASSF1A association site similar to that found on MOAP-1 (Figure 4). However, PNMA1 does not associate with either Bax or RASSF1A [73], and, therefore, although unknown, the mechanism by which it induces cell death presumably differs from MOAP-1. It remains to be determined how, and if, MOAP-1 may impinge on the pathogenesis of paraneoplastic syndromes.

10. Concluding Remarks

MOAP-1 is a highly regulated pro-apoptotic molecule that demonstrates multiple potential properties of a candidate tumor suppressor protein. Given that MOAP-1 regulates RASSF1A pro-apoptotic function and RASSF1A is also epigenetically silenced in a large number of human cancers, it is possible that the combined loss of MOAP-1 and RASSF1A during carcinogenesis may result in the inhibition of extrinsically activated cell death signaling pathways in cancer cells. RASSF1A has now been demonstrated to influence several other biological processes such as cell cycle, microtubule dynamics, and cell migration. Therefore, it will be interesting to explore which of these biological processes MOAP-1 may also be involved in and that may be important for it to behave as a potential tumor suppressor protein.

Abbreviations

AICAR: 5-Aminoimidazole-4-carboxamideribosideor acadesine
Bcl-2: B-cell lymphoma-2
BH3: Bcl-2 homology 3
FTY720: 2-Amino-2[2-(4-octylphenyl) ethyl]-1,3-propane-diol hydrochloride
LATS1: Large tumor suppressor, homolog 1
MOAP-1: Modulator of apoptosis
MST: Mammalian sterile 20-like
pI: Isoelectric point
PND: Paraneoplastic neurological disorder
PNMA: Paraneoplastic Ma antigen
RAS: Rat sarcoma
RASSF: Ras association domain family
RA: Ras association domain
SNP: Single nucleotide polymorphism
TNFα: Tumor necrosis factor α
TNF-R1: Tumor necrosis factor receptor 1
TRAIL: TNF-related apoptosis-inducing ligand
TRIM39: Tripartite motif containing 39
YAP1: Yes-associated protein 1.

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Review Article

RASSF1 Polymorphisms in Cancer

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Ras association domain family 1A (RASSF1A) is one of the most epigenetically silenced elements in human cancers. Localized on chromosome 3, it has been demonstrated to be a bone fide tumor suppressor influencing cell cycle events, microtubule stability, apoptosis, and autophagy. Although it is epigenetically silenced by promoter-specific methylation in cancers, several somatic nucleotide changes (polymorphisms) have been identified in RASSF1A in tissues from cancer patients. We speculate that both nucleotide changes and epigenetic silencing result in loss of the RASSF1A tumor suppressor function and the appearance of enhanced growth. This paper will summarize what is known about the origin of these polymorphisms and how they have helped us understand the biological role of RASSF1A.

1. Introduction

Cancer is a disease affecting 1 in 3 adults worldwide and is considered to be the second leading cause of death in both Canada and the United States behind heart disease [1, 2]. It is thought that cancer arises due to the occurrence of 2–5 genetic events to potentiate tumor formation and sustain abnormal growth [3]. These genetic changes occur in passenger genes (to support the cancer phenotype) and driver genes (to promote the cancer phenotype) [4]. About 10% of driver genes code for oncogenes that promote accelerated growth. However, about 90% of the driver genes code for tumor suppressor genes that inhibit accelerated growth [3], suggesting that tumor suppressor genes play an integral part in the origin of cancer. Evidence also suggests that the mutation rate of tumor suppressor genes are much higher than oncogenes supporting their importance in cancer formation [3, 5].

In 2000, Hanahan and Weinberg systematically described several key features or "hallmarks" of cancer that defined the behavior of a cancer cell [6]. These defining features of a cancer cell included the unique properties of limitless replicative potential, evasion of apoptosis, ability to stimulate neo-vascularization, invasion and metastasis, inhibition of suppressor pathways, and sustained proliferation. As described in their seminal paper, the aforementioned hallmarks are acquired through a "multistep process" that allows the cancer cells to acquire key survival traits while avoiding the watchful eye of established molecular "checkpoints" to inhibit abnormal growth [7]. It was around this time that the RASSF1 was identified as a potential tumor suppressor gene on chromosome 3, at 3p21.23 [8, 9]. Now more than a decade later, RASSF1A has been demonstrated using numerous approaches to be a tumor suppressor gene and an important driver gene in cancer influencing/intersecting with many of the hallmarks of cancer [8, 10]. It is epigenetically silenced in the majority of cancers by promoter specific methylation, resulting in loss of expression of the RASSF1A protein [11]. Although expression loss of RASSF1A by methylation occurs frequently in cancer, nucleotide changes by somatic mechanisms have also been detected in patients from several cancer subtypes. Several studies have tried to elucidate the importance of these polymorphic changes and how it may affect the tumor suppressor function of RASSF1A. They

have also revealed interesting and surprising influences on numerous aspects of biology.

2. The Origin of RASSF1A Polymorphisms

The RASSF1 gene consists of eight exons alternatively spliced to produce 8 isoforms, RASSF1A-H, that have distinct functional domains including the Ras association (RA) domain [9, 14]. Of these, RASSF1A and RASSF1C are the predominant ubiquitously expressed forms in normal tissues [9, 11]. RASSF1C has been demonstrated to be perinuclear in appearance in NCI H1299 lung cancer cells [15], nuclear in HeLa cells with translocation to the cytosol upon DNA damage [16], and localized to microtubules in a similar fashion to RASSF1A in 293T cells [17, 18]. Thus, the localization of RASSF1C is varied and controversial. This is not the case for RASSF1A as it has been demonstrated by our group and several others to be a microtubule binding protein having a microtubule like localization and functioning to stabilize tubulin in a taxol like manner [13, 18, 19]. To date, a crystal structure for RASSF1A or RASSF1C has not been identified, but Foley et al. [20] provided a molecular model of the N-terminal C1 domain containing four zinc finger motifs which is very similar to the one found on RASSF5A/Nore1A [21]. The zinc finger motifs have now been demonstrated to be involved in death receptor associations and possible associations with other receptors or signaling components [20]. In addition to the C1 domain, RASSF1A has been noted to have a sequence specificity motifs to associate with SH3 domain (PxxP); motifs for 14-3-3 associations; a Ras association (RA) domain (although association is weak or indirect for K-Ras) [10]; associations with the anaphase promoting complex protein cdc20 and the autophagy protein C19ORF5/MAP1S; and heterotrophic associations with the Hippo proapoptotic kinase (MST1/2) and the BH3-like protein modulator of apoptosis 1 (MOAP-1) through the Salvador/RASSF/Hippo (SARAH) domain (both reviewed elsewhere in this issue) (please see Figure 1 for schematic summary of RASSF1A protein associations).

RASSF1A polymorphisms have been identified in several cancers as listed in Table 1 and can be mapped to specific protein interaction domains (Figure 1). These polymorphisms have been found in tumors from numerous cancer patients and cell lines [22]. The population distribution and significance of these alterations in tumorigenesis remain to be determined but do vary from 9% to 33% of the specific cancer population. The majority of RASSF1A polymorphisms have been confirmed using several approaches as outlined by the 1000Genome project (http://www.1000genomes.org/), HapMap project (http://hapmap.ncbi.nlm.nih.gov/) and submitted by multiple sources (Table 1 and NCBI SNP database [http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?showRare=on&chooseRs=coding&go=Go&locusId=11186] and University of Maryland SNP database [http://bioinf.umbc.edu/dmdm/gene_prot_page.php?search_type=gene&id=11186, NP_009113]). Recently, a comprehensive study of 400 lung, renal, breast, cervical, and ovarian cancers by Kashuba et al. [22] revealed frequent loss of genetic material on chromosome 3p in 90% of the tumors investigated. Furthermore, they determined that the mutation rate in cancer for RASSF1A was 0.42 mutation frequency/100 base pair whereas in the "normal" population was about 0.10 mutations/100 base pairs. They speculate that RASSF1A has a 73% GC content within exons 1–2 which may explain the high mutation rate of RASSF1A within cancer cells. Within cell lines, RASSF1A was found to carry 0.7 mutations/100 bp in the Burkitt's lymphoma-derived cell lines, BL2 and RAMOS, whereas it was 0.14 in the renal carcinoma cell line KRC/Y and, with each division of the BL2 lymphoma line, transitional mutations were observed. Interestingly, codon changes in RASSF1A were also observed in 15 normal human hearts that included two nucleotide changes (CTA to CTG and GTA to GTG) but no amino acid changes [22]. They speculate that RASSF is simply located in an area that is "extensively damaged" and susceptible to mutational pressures in 90% of epithelial cancers [22].

The most common polymorphism is the alanine (A) to serine (S) at amino acid 133 (A133S) located within the ATM DNA damage checkpoint kinase site (please see below sections). This has been identified as a single nucleotide germ line polymorphism (SNP) on both alleles in some breast cancer patients and is significantly associated with BRAC1/2 mutations. Patients with wild-type BRAC1/2 and RASSF1A A133S have a +15-year better survival period than those harboring both BRAC1/2 mutations and RASSF1A A133S [23, 29]. The RASSF1A A133S SNP has been found in 20.6% of patients with breast carcinomas [23, 29], 19.8% in lung cancer [29, 32], 11.1% in head and neck cancer [32], 6.9% in colorectal cancer [32], 14.3% in esophageal cancer [32], 24.3% in patients with fibroadenoma and in 2.9%–10% of healthy controls [23, 32]. Interestingly, Gao et al. [29] also revealed the presence of the A133S polymorphism in brain and kidney cancer patients and Bergqvist et al. (2010) detected the presence of the A133S SNP in 18.4% of the white British female population [28]. The high percent obtained for the latter is surprising and requires further validation. The prevalence of the rest of the RASSF1A polymorphisms has not been determined yet, and functional studies to systematically determine influence of these polymorphisms on RASSF1A biological function are yet to be done. However, in this paper we will only summarize what has been carried out already to ascertain the consequences of polymorphisms to RASSF1.

3. RASSF1A: A Key Element in Cellular Stability

One of the most striking features of RASSF1A is its microtubule appearance. Numerous tagged versions of RASSF1A have all revealed similar microtubule-like appearance as seen in MCF-7 breast cancer cells in Figure 2. This appearance has been observed in many other cell lines with similar appearances. It has also been determined that both N- and C-terminal residues of RASSF1A are required for the microtubule appearance of RASSF1A [13, 18]. Several groups have characterized the appearance and function of the microtubule localization of RASSF1A. It has been demonstrated that the microtubule localization of RASSF1A mainly

Table 1: RASSF1A single nucleotide polymorphisms. Several RASSF1A polymorphic changes have been identified as outlined in Table 1. SNP sites consulted to draft this table include NCBI (at http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?showRare=on&chooseRs=coding&go=Go&locusId=11186) and DMDM (at http://bioinf.umbc.edu/dmdm/gene_prot_page.php?search_type=gene&id=11186).

Polymorphism	Tissue or cell line origin	SNP ID[%] and other information	References
K21Q (AAG → CAG)	Breast (tumor) Kidney (renal carcinoma cell TK10 and KRC/Y) Lung (Non small cell Lung cancer cell line)	rs4688725[*,**,#,]	Schagdarsurengin et al. [23]; Dammann et al. [24]; Agathanggelou et al. [25]; Burbee et al. [26]
R28H (CGT → CAT)	Breast (Tumor) Lung (nonsmall cell lung cancer cell line)	Presence in lung carcinomas are rare	Schagdarsurengin et al. [23]; Dammann et al. [24]; Burbee et al. [26]
V47F (GTC → TTC)	Not listed	rs61758759[*,**,#,]	NCB1[%]
R53C (CGC → TGC)	Breast (tumor) Lung (nonsmall cell lung cancer cell line)	Q9NS23[$]	Schagdarsurengin et al. [23]; Dammann et al. [24]; Burbee et al. [26]
A60T (GCA → ACA)	Breast	No SNP ID found	Agathanggelou et al. [25]
C65R (TGC → CGT)	Breast (tumor)	No SNP ID found	Dallol et al. [27]
S131F (TCT → TTT)	Breast (tumor) Kidney (Wilm's tumor)	No SNP ID found	Schagdarsurengin et al. [23]; Dammann et al. [24]
A133S (GCT → TCT)	Breast (tumor, fibroademonas), 33% Kidney (Wilm's tumor), 21% Brain (medulloblastoma), 9% Muscle (rhadomyosarcoma), 19% Lung (nonsmall cell lung cancer cell line)	rs52807901 and rs2073498 Association with BRAC1/2 mutations Homozygous in breast cancer 21% of kids with germ line mutation, maternal in origin	Schagdarsurengin et al. [23]; Dammann et al. [24]; Bergqvist et al. [28]; Gao et al. [29]; Burbee et al. [26]; Lusher et al. [30]
I135T (ATT → ACT)	Lung (nonsmall cell lung cancer cell line) Breast (tumor cell line)	No SNP ID found	Dammann et al. [24]; Agathanggelou et al. [25]
V211A (GTC → GCC)	Breast	No SNP ID found	Agathanggelou et al. [25]
R201H (CGC → CAC)	ENT (nasopharyngeal carcinoma)	In 23 tumor samples, 34 other polymorphisms were detected (not listed in this table) with 30 transitions, 2 transversions, and 2 deletions (6 in SH3/C1 domain and 6 in RA domain)	Zhi-Gang Pan et al. [31]
E246K (GAA → AAG)	Breast (tumor)	No SNP ID found	Agathanggelou et al. [25]
R257Q (CGG → CAG)	Breast (Tumor) Lung (nonsmall cell lung cancer cell line	No SNP ID found	Schagdarsurengin et al [23]; Dammann et al. [24]; Agathanggelou et al. [25]; Dallol et al. [27]
H315R (CAC → CGC)	NCBI SNP database, source unknown	rs52792349 and rs12488879	Geoffery Clark (personnel communication)
Y325C (TAT → TGT)	Breast (tumor) Lung (nonsmall cell lung cancer cell line	No SNP ID found	Schagdarsurengin et al. [23]; Dammann et al. [24]; Burbee et al. [26]
L270V (CTG → GTG)	Cervical (tumor)	No SNP ID found	Schagdarsurengin et al. [23]; Dammann et al. [24]
A336T (GCC → ACC)	Lung (nonsmall cell lung cancer cell line	No SNP ID found	Dammann et al. [24]

[%] http://bioinf.umbc.edu/dmdm/gene_prot_page.php?search_type=gene&id=11186.
[$] UniProtKB/Swiss-Prot.
[*] Validated by multiple, independent submissions to the refSNP cluster.
[**] Validated by frequency or genotype data: minor alleles observed in at least two chromosomes.
[#] Validated by the 1000 Genomes Project, http://www.1000genomes.org/.

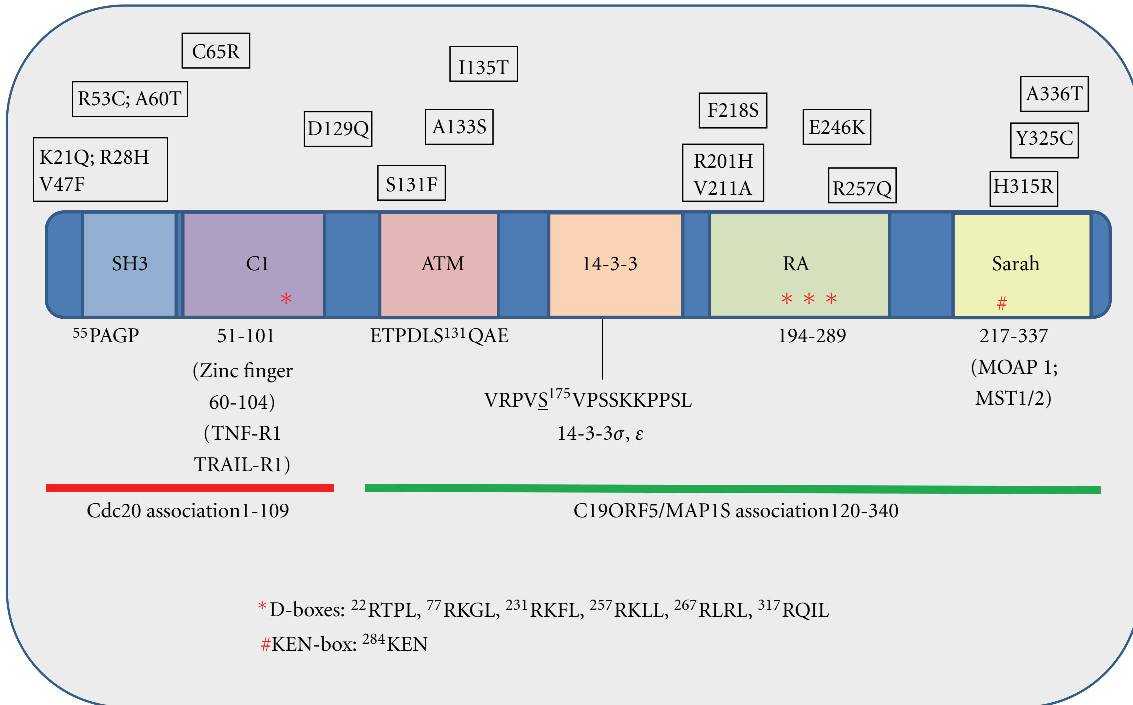

Figure 1: Schematic of RASSF1A with location of identified polymorphisms. Location of identified RASSF1A polymorphisms is indicated with respect to amino acid location, changed amino acid, and exon location. A potential binding sequence to an SH3 domain has been identified with a PxxP motif. The ATM phosphorylation site is underlined with surrounding residues shown. The docking sites for several RASSF1A effector proteins are shown including the location of potential D- and KEN-boxes for protein association (D1 to D6). The latter boxes are thought to be important for associations with APC/cdc-20 [12]. The Ras association domain (RA) is present in RASSF1A but has not been convincingly demonstrated to associate with the Ras family of oncogenes [10]. The SARAH domain modulates heterotypic associations with the sterile-20-like kinases, MST1 and MST2 (adapted from El-Kalla et al. (2010)) [13] and Gordon and Baksh (2011) [10].

functions to stabilize tubulin both in interphase and in mitosis even in the presence of the microtubule destabilizer, nocodazole [13, 27, 33, 34]. To date, RASSF1A has not been demonstrated to colocalize to actin or intermediate filaments. RASSF1A associations function to stabilize tubulin in a paclitaxel (taxol)-like manner [17, 27] especially during mitosis allowing sister chromatid segregation. This function is governed by associations with γ-tubulin at spindle poles and centromeric areas during metaphase and anaphase and near the microtubule organizing center (MTOC) where microtubules emerge and nucleate [35–37]. If the microtubule spindle complex is not properly formed, cell death proceeds to prevent inheritable aneuploidy. In the absence of cell death pathways chromosomal missegregation and inheritable aneuploidy arise which can lead to malignancy. Several of the effects on microtubule biology have been observed in mouse embryonic fibroblasts (MEFs) obtained from *Rassf1a*$^{-/-}$ mice developed by two separate groups [19, 38]. *Rassf1a*$^{-/-}$ mice are viable, fertile and retain expression of isoform 1C. However, by 12–16 months of age they have increased tumor incidence, especially in the breast, lung, and immune system (gastrointestinal carcinomas and B-cell-related lymphomas) [19, 38]. These data suggest a tumor suppressor function specific for the RASSF1A isoform. MEFs obtained from *Rassf1a*$^{-/-}$ mice are more susceptible to nocodazole-induced microtubule depolymerization suggesting a protective effect of RASSF1A on microtubule stability similar to what has been observed using tissue culture approaches [19].

It has now been demonstrated that RASSF1A disease associated polymorphisms may affect the function of RASSF1A as a microtubule stabilizer. It was demonstrated that the S131F mutant of RASSF1A continued to maintain the ability to promote tubulin stability as determined by immunofluorescence microscopy and acetylation status of tubulin [17]. Furthermore, it was demonstrated by Vos et al. [17] that RASSF1C could not function in a similar manner to RASSF1A to stabilize tubulin. This provided one of the first evidences for differential function for these two prominent isoforms of the RASSF1 loci. A comprehensive analysis of several other RASSF1A polymorphisms was carried out by Liu et al. [39]. They demonstrated that polymorphisms around the ATM phosphorylation site (A133S, S131F, and I135T) maintained the microtubule appearance of RASSF1A. A second comprehensive study revealed that the C65R and R257Q polymorphisms of RASSF1A resulted in “atypical localizations” of RASSF1A away from a microtubular appearance [27]. Furthermore, both C65R (a residue within the C1 domain) and R257Q (a residue within the RA domain) promoted enhanced BrdU incorporation into NCI-H1299

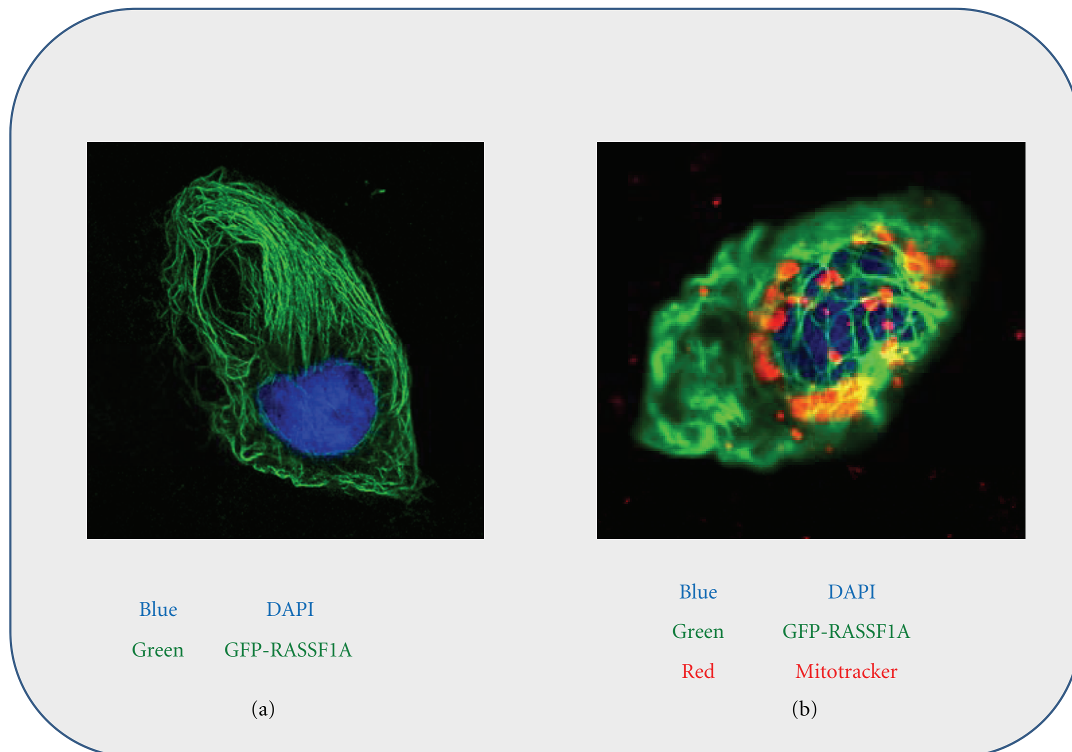

Figure 2: Microtubule localization of RASSF1A. GFP-RASSF1A was expressed in U2OS osteosarcoma cells (a and b) and costained with DAPI to reveal the nucleus (a and b) and with mitotracker red to reveal mitochondrial localization (b). Areas of yellow reveal colocalization and all images were acquired using confocal microscopy using a Zeiss system and a 63x oil immersion lens.

nonsmall cell lung cancer cells suggesting loss of tumor suppressor function. Recently, we have also observed a complete loss of the microtubule localization of RASSF1A in the presence of a C65R change and "oncogenic" properties of this polymorphism in a classical xenograft assay in athymic mice [13]. The C65R polymorphism acquired a nuclear localization for unexplained reasons and also failed to stabilize tubulin in the presence of the microtubule depolymerizing agent nocodazole [13]. It clearly lost the tumor suppressor function of RASSF1A in a xenograft assay and can robustly drive enhanced growth [13]. Similarly, both the A133S and E246K mutants maintained microtubule localization and lost tumor suppressor function but not to the level of the C65R polymorphism (xenograft assays were carried out in HCT116 colon cancer cells) [13]. We are currently characterizing many of the other polymorphisms in Table 1 for their ability to behave as tumor suppressor, inhibit abnormal growth, and affect microtubule stability and protein interaction with established RASSF1A effectors.

Interestingly, it has been reported that Epstein-Barr virally encoded protein, latent membrane protein 1 (LMP1) can function to transcriptionally decrease RASSF1A levels and promote tubulin depolymerization and mitotic instability in human epithelial cells (HeLa and HaCaT) [40]. Punctuate structures of tubulin were observed in the cytoplasm indicative of tubulin depolymerization [40]. Decreased RASSF1A levels resulted in increased phosphorylation of IκBα and elevated NFκB activity. Cause and effect of changes in NFκB activity were not fully elucidated. However, we have evidence that the loss of RASSF1A can lead to enhanced NFκB activity (El-Kalla et al., unpublished observations) suggesting that the decreased expression of RASSF1A induced by LMP1 production may have resulted from the loss of the ability of RASSF1A to restrict NFκB function. EPV infection is closely related to the appearance of nasopharyngeal cancers and we speculate that a precondition characterized by enhanced NFκB activity (and hence inflammation) may promote tumorigenesis and the appearance of nasopharyngeal cancers upon EBV infection. We are currently exploring the role of RASSF1A as a molecular link between inflammation and tumorigenesis.

4. RASSF1A: Linking Extrinsic Death Receptor Stimulation to Bax Activation

Every cell has an inherent ability to die under abnormal conditions. This ability has been programmed by nature into every cell and follows a defined series of events. Apoptosis is critical for multiple physiological processes, including organ formation, immune cell selection, and inhibition of tumor formation [41]. Two types of signaling pathways promote apoptosis using the mitochondria. The "intrinsic" pathway is activated by noxious factors such as DNA damage, unbalanced proliferative stimuli, and nutrient or energy depletion. Components of intrinsic-dependent apoptosis are still unclear, although Bcl-2-homlogy domain 3 (BH3) proteins are required. In contrast, the "extrinsic" pathway is stimulated by specific death receptors (e.g., TNFα receptor R1 (TNF-R1), TNFα-related apoptosis-inducing ligand receptor (TRAIL-R1) or Fas (CD95)) [42–44]. Molecular mechanisms modulating programmed cell death (apoptosis) impinge on growth and immune cell function. We speculate that these cellular processes may be regulated in part by tumor suppressor pathways, pathways frequently inactivated in several disease states (such as cancer and autoimmune/inflammatory disorders).

RASSF1A is one element involved in death receptor-dependent cell death that is epigenetic-silenced in numerous cancers. In the majority of these studies, RASSF1A epigenetic silencing strongly correlates with the epigenetic silencing of three other genes—p16^{INK4a}, death associated protein kinase (DAPK), and caspase 8 [45–48]. Two of these genes are involved in proapoptotic pathways, DAPK and caspase-8 [43, 49, 50]. DAPK is a unique calcium/calmodulin activated serine/threonine kinase involved in several cell death-related signaling pathways including tumor necrosis factor α receptor 1 (TNF-R1) cell death and autophagy [50, 51]. It is a tumor suppressor protein [50] that has also been demonstrated to be involved in associations with and the regulation of pyruvate kinase, a key glycolytic enzyme that may be influential in the link between metabolism and cancer [52]. We have evidence to demonstrate association of RASSF1A and DAPK (Baksh et al., unpublished observations) and RASSF1A has two potential phosphorylation sites for DAPK within the RA domain at ^{193}GRGTSVRRRTSFYLPK [53]. Curiously, these sites have also been demonstrated to be sites for protein kinase C [54] and aurora kinases [55]. In the presence of S197A or S203A mutant of RASSF1A, PKC failed to phosphorylate RASSF1A resulting in the loss of microtubule organization in COS-7 cells. Similarly, Aurora B kinase failed to phosphorylate RASSF1A in the presence of S203A resulting in a failed cytokinesis [55]. It remains to be determined the physiological importance of these potential DAPK phosphorylation sites.

Caspase 8 is cysteine-dependent aspartate-directed protease and an initiator caspase, and targeted activation of caspase 8 is driven by the disc inducing signaling complex (DISC) [43, 49]. DISC-dependent activation of caspase 8 triggers a series of events resulting in the cleavage of Bid and insertion of Bid on the outer mitochondrial membrane, the release of small molecules (such as cytochrome c) from the mitochondria into the cytosol, and the activation of downstream effector caspases (such as caspase-3) [56]. Intrinsic pathway stimulation also leads to cytochrome c release and effector caspase activation. Once activated, effector caspases cleave several proteins (such as poly(ADP-ribose) polymerase (PARP)) and activate specific DNA endonucleases resulting in nuclear and cytoplasmic breakdown [57].

Our research group was the first to define and continues to define some of the molecular mechanisms of death receptor-dependent apoptotic regulation by RASSF1A [20, 58, 59]. Ectopic expression of RASSF1A (but not RASSF1C) specifically enhanced death receptor-evoked apoptosis stimulated by TNFα that does not require caspase 8 activity or Bid cleavage [20, 58]. We have also shown that RASSF1A does not influence the intrinsic pathway of cell death [58]. Furthermore, we demonstrated that microtubule localization was required for association with death receptors and for the role of RASSF1A in apoptosis [13, 20]. In contrast, RASSF1A knockdown cells (by RNA interference) and *Rassf1a*$^{-/-}$ knockout mouse embryonic fibroblasts (MEFs) have significantly reduced caspase activity, defective cytochrome c release and Bax translocation (but not Bid cleavage), and impaired death receptor-dependent apoptosis [58]. These data suggest a direct link of death receptor activation of Bax through RASSF1A. Our current model of RASSF1A-mediated cell death is described in Figure 3. Death receptor stimulation functions to bring RASSF1A (and not RASSF1C or RASSF5A/Nore1A) and modulator of apoptosis 1 (MOAP-1) to TNF-R1 in order to promote a more "open" MOAP-1 to subsequently associate and promote Bax conformational change and translocation to the mitochondria to activate cell death (Figure 3) [20, 58–60]. We have evidence that the 14-3-3 may keep RASSF1A in check and inhibit it from promoting cell death or associating with other unexplored signaling components [59]. We are currently characterizing the primary and secondary signals required for MOAP-1 induced Bax conformational change and the apoptotic regulation of MOAP-1 by ubiquitination (Law et al., unpublished observations).

To date, very little is known about the cell death properties of numerous RASSF1A polymorphisms. Dallol et al. demonstrated that both C65R and R257Q promoted enhanced BrdU incorporation into NCI-H1299 non-small cell lung cancer cells suggesting loss of tumor suppressor function and possible loss of cell death properties [27]. We have observed partial activation of apoptosis in the presence of several RASSF1A polymorphisms (such as C65R, A133S, I135T, and A336T) suggesting importance to death receptor-dependent apoptosis via ATM site and SARAH domain associations (El-Kalla et al., unpublished observations). Further analysis is warranted to explore how RASSF1A polymorphisms may affect death receptor-dependent apoptosis.

Although not discussed in great detail here, RASSF1A can also promote cell death utilizing the autophagic protein, C19ORF5/MAP1S, [27, 33, 61] the Hippo pathway components MST1/2 and possibly Salvador [14, 62], and, in melanoma cells, influence Bcl-2 levels and activate apoptosis signal regulating kinase 1 (ASK-1) [63]. Min et al. [33] demonstrated that the ability of RASSF1A to efficiently

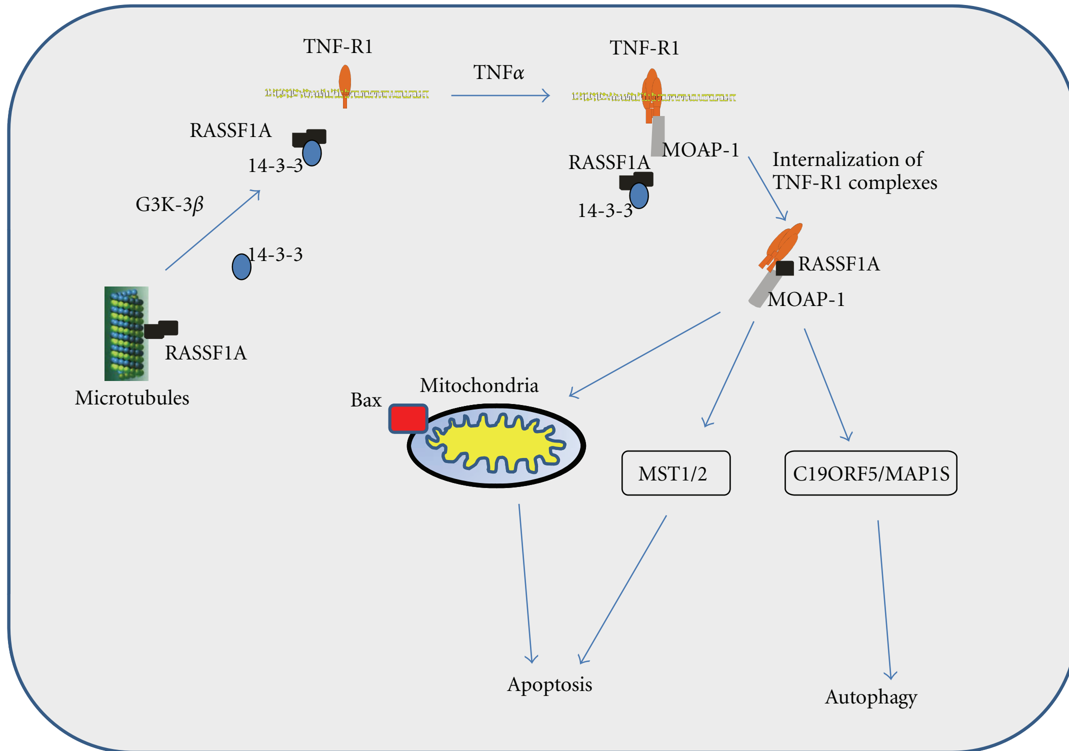

Figure 3: Model for the RASSF1A/MOAP-1 proapoptotic pathway. Death receptor-induced cell death (TNFα is used as an example) can result in the recruitment of protein complexes to activate Bax and promote apoptosis. Basally, RASSF1A is kept complexed with 14-3-3 by GSK-3β phosphorylation in order to prevent unwanted recruitment of RASSF1A to death receptor and uncontrolled stimulation of Bax and apoptosis. Once a death receptor stimuli have been received (TNFα as shown above), the TNF-R1/MOAP-1/RASSF1A complex promotes the "open" form of MOAP-1 to associate with Bax. This in turn results in Bax conformational change and recruitment to the mitochondria to initiate cell death. Following release from TNF-R1/MOAP-1 complex, RASSF1A may reassociate with 14-3-3 to prevent continued stimulation of this cell death pathway (unpublished observations). Please see text for further details.

inhibit APC/cdc20 activity during mitosis (please see next section) is dependent on the recruitment of RASSF1A to spindle poles via C19ORF5/MAP1S. C19ORF5/MAP1S was also shown to regulate mitotic progression by stabilizing mitotic cyclins in a RASSF1A-dependent manner. Recently, C19ORF5/MAP1S was demonstrated by Lui et al. [61] to associate with a component of the autophagosome, LC3, and the mitochondria-associatedv leucine-rich PPR-motif containing protein (LRPPRC) protein. These associations suggest that C19ORF5/MAP1S may serve as a potential link between autophagic cell death, mitochondria, and microtubules and appears to require RASSF1A. It will be essential to determine associations of RASSF1A polymorphisms with key cell death mediators, such as MOAP-1, TNF-R1, DAPK, C19ORF5/MAP1S, and MST1/2 in order to ascertain their importance in influencing the tumor suppressor function of RASSF1A. A detailed discussion about the Hippo and RASSF1A/MOAP-1 pathways of cell death is presented in this special review.

5. Cell Cycle Control Pathways Influenced by RASSF1A

As mentioned previously, RASSF1A is a microtubule binding protein that colocalizes with α- and β-tubulin, and with γ-tubulin on centromeres [35–37]. RASSF1A is thought be an important component of mitotic spindles and can influence the separation of sister chromatids at the metaphase plate. This observation has held true five years later and reinforced the findings of Song et al. [64] of the possible involvement of RASSF1A in cell cycle control. Although, very limited knowledge of the cell cycle effects of polymorphic forms of RASSF1A are known, several lines of evidence do suggest a role in cell cycle control. In 2004, RASSF1A was identified as an interacting protein with the anaphase promoting complex (APC)/cdc20 and prevented the ability of APC/cdc20 to degrade cyclins A and B in order to exit mitosis [64]. In the absence of RASSF1A, cyclins A and B were rapidly degraded due to increased ubiquitination of the cyclins to allow exit from mitosis.

Whitehurst et al. [65] supported this role for RASSF1A and further identified β-TrCP as associating with RASSF1A and functioning to restrict the role of APC-cdc20 in mitotic progression. β-TrCP is an IκBα E3 ligase and negative regulator of the β-catenin/WNT signaling pathway. Although Liu et al. could not find evidence for a RASSF1A-APC/cdc20 association [66], the influence of RASSF1A on APC/cdc20 was once again demonstrated by Chow et al. in 2011 [67]. They not only demonstrated an association with APC/cdc20, but also clearly showed that a "RASSF1A-APC/cdc20 circuitry" was in place in HeLa cells to regulate mitosis. RASSF1A associates with APC/cdc20 via two D boxes at the N-termini (DB1 and DB2) and keeps it inhibited until there is mitotic activation of the serine/threonine kinases Aurora A/B. Phosphorylation of RASSF1A by Aurora A/B on T202 or S203 subsequently labels RASSF1A as a target to the E3-ubiquitin ligase activity of APC, ensuring that mitosis proceeds by degrading RASSF1A and suppressing its mitotic inhibitor function [12]. They speculate that this occurs before spindle body formation and sister chromatid separation. Their results are intriguing and reveal the complex signaling world that RASSF1A is part of.

Beyond a RASSF1A-APC/cdc20 molecular control of mitosis, research has continued into a potential role of RASSF1A during cell cycle progression. This has led to several observations suggesting RASSF1A G1/S regulation of cyclin D1 [63, 65, 68] in melanoma and HeLa cells (resp.), interaction with the transcriptional regulator $p120^{E4F}$ at the G1/S phase transition resulting in inhibition of passage from G1 [69], DNA damage control regulation by ATM and by the DNA damage binding protein 1 (DDB1) that can associate with RASSF1A linking to the E3-ligase cullin 4A during mitosis [70]. The $p120^{E4F}$ transcription factor was determined to be involved in inhibiting the transcription of cyclin A, resulting in the failure of cyclin A to associate with CDK2 to allow for progression through S phase. RASSF1A cooperates with $p120^{E4F}$ to repress cyclin A expression by enhancing its binding at the promoter region [69]. ATM and DDB1 are important DNA damage control elements during ultraviolet and gamma irradiation which have evolved to repair damage DNA and will be discussed elsewhere in this special RASSF issue.

Shivakumar et al. revealed that in both H1299 non-small cell lung cancer and in the human mammary epithelial telomerase immortalized (HME50-hTERT) cell line, over-expression of RASSF1A wild-type expression construct can reduce BrDU accumulation and cyclin D1 expression [68]. The ability of RASSF1A to inhibit growth, and cyclin D1 expression was lost in the presence of the A133S and S131F ATM site mutants of RASSF1A suggesting an important role in tumor suppression [68]. Other polymorphic forms of RASSF1A have not been explored with respect to their abilities to regulate mitosis. What these studies reveal is how highly regulated RASSF1A is, not only in interphase cells, but especially in cells undergoing active cell division. It can then be appreciated how devastating the functional consequence of the loss of RASSF1A would be resulting in an unregulated and unwanted increase in mitotic cyclins, accelerated mitosis, enhanced growth and tumor formation. It would be interesting to speculate that they may result in the loss of the ability of RASSF1A to properly regulate mitosis and inhibit unwanted proliferation. It is imperative that we understand completely how polymorphic changes in RASSF1A may influence the important role of RASSF1A in mitosis and other biological pathways (Figure 4).

6. The DNA Damage Connection

One of the first motifs identified on RASSF1A was the phosphorylation site for the DNA damage serine/threonine kinase Ataxia telangiectasia mutated (ATM). ATM is usually activated and recruited in response to double strand breaks. It is part of a DNA damage checkpoint that ensures that damaged DNA is repaired in a timely and efficient manner. RASSF1A has been shown by several groups to be phosphorylated by ATM and the ATM site polymorphisms are present in several cancer types [71]. Although not currently well defined, RASSF1A is believed to have an important role in DNA damage control as evidenced by associations with xeroderma pigmentosum complementation group A (XPA) [72] and phosphoregulation by ATM [71, 73]. XPA is involved in nucleotide excision repair and association with RASSF1A has only been identified in a yeast two-hybrid screen [24]. Hamilton et al. [71] elucidated a novel pathway linking ATM-dependent phosphorylation of RASSF1A in response to gamma irradiation on serine-131 followed by MST/LATS activation resulting in Yes associated protein (YAP)/p73-dependent transcriptional program to promote cell death. The S131F mutant of RASSF1A lacked the ability to carry out the transactivation of YAP/p73. Curiously, RASSF1C has been demonstrated to be constitutively anchored to the death domain-associated protein (DAXX) in the nucleus and is released upon UV-induced DNA damage [16]. Localization with DAXX occurs on promyelocytic leukaemia-nuclear bodies (PML-NBs). DNA damage promotes the degradation and ubiquitination of DAXX, release of RASSF1C to allow the nucleocytoplasmic shuttling of RASSF1C to cytoplasmic microtubules, and the activation of the SAPK/JNK pathway in HeLa cells. RASSF1A was shown to only associate weakly with DAXX suggesting a specific role for RASSF1C [16]. Recently, it was demonstrated that the E3 ligase, Mule, can ubiquitinate RASSF1C under normal conditions, and both Mule and β-TrCP can ubiquitinate RASSF1C under UV exposure [74]. These studies and others have continued to demonstrate the diverse role that the splice variants of RASSF1 may function in biology. A detailed discussion about the role of RASSF1A during DNA damage repair will be presented in this special review.

7. RASSF1C: The Other RASSF1 Isoform

Very little is known about the biological role for the other major splice variant of the RASSF1 gene family. Several lines of evidence suggest that RASSF1C may be a tumor suppressor gene in prostate and renal carcinoma cells but not in lung cancer cells [75]. In fact, it has been demonstrated by Amaar et al. that the loss of RASSF1C actually results in the loss of proliferation of lung and breast cancer cells suggesting a prosurvival (not tumor suppressor) role for

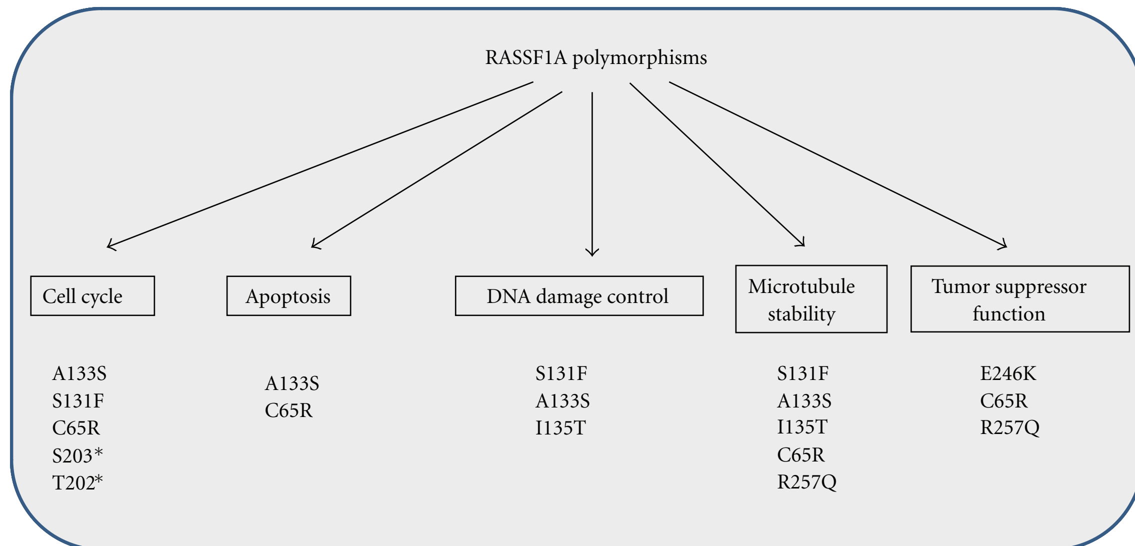

Figure 4: Identified biological roles for RASSF1A polymorphisms. Several polymorphisms have been identified for RASSF1A over the past decade since it was first cloned. Biological analyses of the *in vivo* role have identified the importance of RASSF1A over numerous pathways. This figure summarizes what is known about RASSF1A polymorphisms. *denotes a nonpolymorphic but mutational change. This change does not naturally exist in the cancer patient population to our knowledge.

RASSF1C [76, 77]. Furthermore, RASSF1C can associate with the E3 ligase β-TrCP via the $SS_{18}GYXS_{19}$ motif (where X is any amino acid and numbers correspond to amino acid sequence in RASSF1C) at the N-terminus (i.e., not present in RASSF1A) [78] and promote the accumulation and transcriptional activation of β-catenin [78]. Activation of β-catenin would result in enhanced proliferation by transcriptional upregulation of genes such as cyclin D1, Myc, and TCF-1. Thus, either the lack of RASSF1A expression or the overexpression of RASSF1C perturbs β-TrCP E3 ligase/β-catenin homeostasis and WNT signaling pathways.

Unlike RASSF1A, RASSF1C has not been found to be significantly epigenetically silenced in cancer. Polymorphisms to RASSF1C have not been uncovered yet, but a C61F mutation in RASSF1C (equivalent to the S131F mutation in RASSF1A) resulted in the failure of RASSF1C to protect microtubules against nocodazole-induced depolymerization [17]. This would again suggest importance of serine residue within the ATM site found on both RASSF1A and 1C. Recently, it has been suggested that a possible pathogenic role for RASSF1C in cancer may exist as its expression was more than eleven-fold greater in pancreatic endocrine tumors than in normal tissue [79]. It remains to be determined the exact biological role for RASSF1C, but the ability of RASSF1C to function as a tumor suppressor is cell specific and remains to be further investigated and confirmed.

8. The Future of Understanding RASSF Polymorphisms

Knudson stated in 1971 that cancer is the result of accumulated mutations to the DNA of cells and that multiple "hits" to DNA were necessary to cause cancer [80]. It is generally known that the loss of function in a tumor suppressor protein typically requires the inactivation of both alleles of its gene in contrast to proto-oncogenes which promote tumorigenesis due to dominant acting mutations affecting one gene copy. Similar to what Knudson discovered for retinoblastoma, the RASSF1A tumor suppressor may become inactivated by the epigenetic loss by promoter specific methylation of both allele or by a combination of epigenetic silencing and loss of function polymorphic changes. Most cancers investigated to date have >50% of the disease population containing epigenetic silencing of RASSF1A [11, 81]. However, numerous cancers such as cervical, head and neck, myeloma, and leukemia have <25% of the disease population containing epigenetic silencing of RASSF1A. It may be speculated that polymorphic changes to RASSF1A may exist in the latter patients that, in agreement with the Knudson two hit hypothesis, resulting in the loss of function of the RASSF1A tumor suppressor and causing cancer. A systematic and functional analysis of RASSF1A polymorphism is therefore necessary to allow physicians to carry out personalized medicine on patients harboring polymorphic changes to RASSF1A.

Abbreviations

APC: Anaphase promoting complex
ATM: Ataxia telangiectasia mutated
BH3: Bcl-2-homlogy domain 3 (BH3)
DAPK: Death-associated protein kinase
C19ORF5/MAP1S: Chromosome 19 open reading frame 5/microtubule-associated protein 1S
Nore: Novel ras effector
PKC: Protein kinase c
RAS: Rat Sarcoma

RASSF: Ras association domain family
RA: Ras association domain
NFκB: Nuclear factor of kappa light polypeptide gene enhancer in B-cells
MAP: Mitogen activated kinase
MOAP-1: Modulator of apoptosis
SARAH: Salvador/RASSF/Hippo domain
TNFα: Tumor necrosis factor α
TNF-R1: TNFα receptor 1.

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Research Article

Loss of RASSF2 Enhances Tumorigencity of Lung Cancer Cells and Confers Resistance to Chemotherapy

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RASSF2 is a novel pro-apoptotic effector of K-Ras that is frequently inactivated in a variety of primary tumors by promoter methylation. Inactivation of RASSF2 enhances K-Ras-mediated transformation and overexpression of RASSF2 suppresses tumor cell growth. In this study, we confirm that RASSF2 and K-Ras form an endogenous complex, validating that RASSF2 is a bona fide K-Ras effector. We adopted an RNAi approach to determine the effects of inactivation of RASSF2 on the transformed phenotype of lung cancer cells containing an oncogenic K-Ras. Loss of RASSF2 expression resulted in a more aggressive phenotype that was characterized by enhanced cell proliferation and invasion, decreased cell adhesion, the ability to grow in an anchorage-independent manner and cell morphological changes. This enhanced transformed phenotype of the cells correlated with increased levels of activated AKT, indicating that RASSF2 can modulate Ras signaling pathways. Loss of RASSF2 expression also confers resistance to taxol and cisplatin, two frontline therapeutics for the treatment of lung cancer. Thus we have shown that inactivation of RASSF2, a process that occurs frequently in primary tumors, enhances the transforming potential of activated K-Ras and our data suggests that RASSF2 may be a novel candidate for epigenetic-based therapy in lung cancer.

1. Introduction

RASSF2 is a member of the RASSF family of proteins which consists of 10 family members (RASSF1–10). While all the family members are characterized by a conserved RalGDS/AF6 Ras association (RA) domain either in the C-terminal (RASSF1–6) or N-terminal of the protein (RASSF7–10), only RASSF1–6 contain a conserved SARAH (Salvador/RASSF/Hpo) domain adjacent to the RA domain [1–3]. It is well established that RASSF1–6 have tumor suppressor activity, and recent evidence suggests that other members of the family may also function as tumor suppressors [1, 3–8].

Although RASSF2 is structurally related to the better characterized RASSF1A, the mechanisms by which these two family members promote cell death may differ as RASSF2 localizes predominantly to the nucleus [9, 10] whereas RASSF1A is found primarily in the cytoplasm. RASSF2 binds to K-Ras in a GTP-dependent manner [11] and may serve as a K-Ras-specific effector as it forms an endogenous complex with K-Ras [12]. RASSF2 has no apparent intrinsic enzymatic activity or DNA binding properties and thus acts by interacting with other proapoptotic effectors and tumor suppressors, including PAR-4 [13] and the MST1/2 kinases [14, 15], thereby regulating the pathways these effectors control.

Like RASSF1A, RASSF2 is inactivated in a variety of tumors by promoter methylation [8, 9, 11, 13, 16–23]. RASSF2 has the properties of a tumor suppressor in that its overexpression promotes apoptosis and cell cycle arrest *in vitro* and inhibits tumor cell growth and tumor xenograft formation in nude mice [9, 11]. Conversely, loss of RASSF2 expression results in enhanced growth in soft agar and transformation [24]. Loss of RASSF2 may also promote metastasis [23, 25]. RASSF2 may function in additional biological processes other than apoptosis and growth suppression as suggested by *RASSF2* knockout mice. These mice develop normally for the first two weeks after birth, where after they develop growth retardation and die approximately 4 weeks after birth [26]. Additionally, these mice develop

systemic lymphopenia and altered bone development. This suggests that RASSF2 has important functions in early postnatal development and further confirms that RASSF2 has functions distinct from RASSF1A as *RASSF1A* knockout mice develop normally [27, 28].

Although RASSF2 is expressed in a wide variety of tissues [26], its expression is somewhat tissue specific, with the highest levels detected in brain, peripheral blood, and lung [11]. RASSF2 is frequently downregulated in lung cancer [9, 11, 19] with inactivation of RASSF2 being more prevalent in NSCLC than SCLC. K-Ras is frequently mutated in lung cancer [29], and inactivation of RASSF2 enhances the transforming potential of K-Ras in rat kidney cells [24]. Several reports indicate that there is a positive correlation between K-Ras/BRAF mutations and *RASSF2* methylation in primary tumors [21, 24, 30]. Thus, inactivation of RASSF2 confers a growth advantage to tumor cells harboring activated K-Ras, and loss of RASSF2 expression may be a key event in Ras-mediated transformation.

To date, the majority of studies examining the effects of RASSF2 on the transformed phenotype rely on overexpression assays, which although providing useful information, have some drawbacks in that overexpression of proteins from viral promoters may yield expression levels far above physiological levels, thereby generating data that may not be physiologically relevant. We have used RNAi technology to reduce RASSF2 expression levels, a situation that more accurately mimics what occurs in primary tumors, to determine the role of RASSF2 inactivation in transformation. Loss of RASSF2 expression in lung cancer cells dramatically enhanced the transformed phenotype, decreased cell adhesion, and increased invasion. These effects were associated with elevated levels of activated AKT. Furthermore, inactivation of RASSF2 conferred resistance to taxol and cisplatin, suggesting that RASSF2 may be a target for epigenetic therapy in lung cancer.

2. Materials and Methods

2.1. Cell Lines and Culture Conditions. H441 lung cancer cells were maintained in RPMI1640 (Mediatech Inc., Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Mediatech Inc.) and 1% penicillin-streptomycin (Mediatech Inc.).

2.2. Knockdown of RASSF2 by Short Hairpin RNA. H441 cells were transfected with pLKO.1 lentiviral constructs encoding shRNA molecules to RASSF2 with the following sequences: shF2 number 1, 5′-TCTGAAGACCTACAACTTGTA-3′ and shF2 number 2, 5-GCCACCGATTACCCGCTGATT-3′, and a control shRNA that corresponded to RASSF2 sequences but which was ineffective at reducing RASSF2 levels 5′-CCTCCCAAGTAGCTGGAATTA-3′ (Open Biosystems, Lafayette, CO) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and selected with puromycin to obtain a stable bulk population of cells.

2.3. Western Blot Analysis. Total cell lysates were prepared by lysing the cells in RIPA buffer (Sigma, St. Louis, MO) supplemented with 100 μg/mL leupeptin, 100 μg/mL aprotinin, and 1 mM sodium orthovanadate. The lysates were passed through a 21-gauge needle, centrifuged to remove debris, and quantitated using the BioRad Protein Assay (BioRad, Hercules, CA). Equal amounts of protein were resolved on 4–12% NuPage Novex polyacrylamide gels (Invitrogen) and incubated with antibodies against RASSF2 [11], β-Actin (Sigma), phospho-AKT (9271), and AKT (9272) (Cell Signaling Technology, Inc., Danvers, MA). The signal was detected by enhanced chemiluminescence.

2.4. Immunoprecipitation. Endogenous coimmunoprecipitations of Ras and RASSF2 were performed using a Pan-ras antibody conjugated to sepharose beads (Santa Cruz Biotechnology Inc., Santa Cruz, CA) to immunoprecipitate the lysate. The immunoprecipitates were then analyzed by Western Blot using our RASSF2 antibody [11].

2.5. Cell Proliferation Assays. 2×10^5 cells per well were plated in 6-well plates and incubated for 6 days. Cell number was determined each day by counting the number of viable cells in one well of each plate for the different cell lines. Experiments were performed twice in duplicate.

2.6. Cytotoxicity Assays. 5×10^4 cells per well were plated in 12-well plates and incubated with 5 nM taxol, 50 μM cisplatin, or vehicle for 3 days. The number of surviving cells was determined by cell counting. Experiments were performed twice in duplicate.

2.7. Soft Agar Assays. 1×10^4 cells were plated in 6 mL of 0.35% agar in complete growth medium overlaid on a 0.7% agar base, also in complete growth medium. The cells were incubated at 37°C for 2 weeks and resulting colonies were counted after staining for 16 hr with p-iodonitrotetrazolium violet. Experiments were performed twice in duplicate.

2.8. Adhesion Assays. Cell adhesion assays were performed essentially as described [31]. Briefly, 5×10^4 cells per well were plated in BSA-coated 96-well plates and allowed to adhere for 45 min at 37°C. The medium was removed and the adhering cells fixed and stained with crystal violet. The dye was solubilized, and absorbance at 570 nm was used as a measure of adhesion.

2.9. Invasion Assays. 1×10^5 cells per well were plated on a collagen plug in serum-free growth medium in transwell inserts. The inserts were placed in 12-well plates containing complete growth medium and incubated at 37°C for 7 days. Cells on the inner surface of the transwell membrane were removed by scraping with a cotton swab, and cells remaining on the outer surface of the membrane were fixed and stained with crystal violet. The number of cells remaining on the outer surface of the transwell membrane was then quantitated by cell counting.

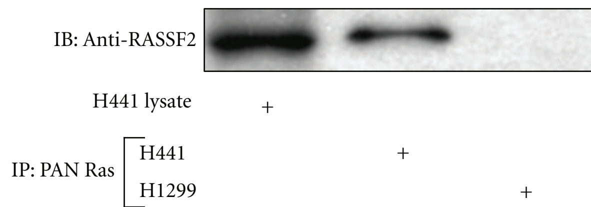


Figure 1: RASSF2 and K-Ras form an endogenous complex. Lysates from H441 and H1299 lung cancer cells were immunoprecipitated with a pan Ras antibody, fractionated on SDS gels, and immunoblotted with an anti-RASSF2 antibody. The endogenous interaction between Ras and RASSF2 was confirmed by the presence of RASSF2 in the proteins precipitated from the H441 cells but not the RASSF2-negative H1299 cells.

3. Results

3.1. RASSF2 Forms an Endogenous Complex with K-Ras. RASSF2 has previously been shown to directly bind to K-Ras *in vitro* in a GTP-dependent manner [11]. To confirm that RASSF2 and K-Ras can form an endogenous complex, we serum-starved then briefly serum-stimulated H441 lung cancer cells that express mutant K-Ras and retain RASSF2 expression [11]. The cells were then lysed and immunoprecipitated with a pan-Ras antibody conjugated to sepharose beads and the immunoprecipitate subjected to Western Blotting with a RASSF2 antibody [11] (Figure 1). The presence of RASSF2 in the immunoprecipitate confirmed that the interaction between RASSF2 and K-Ras is physiologically relevant and RASSF2 is a *bone fide* Ras effector.

3.2. Downregulation of RASSF2 Enhances the Proliferation of Tumor Cells. To determine the biological effects of downregulating RASSF2, we used two independent RASSF2 shRNA constructs to generate stable RASSF2 knockdown cell lines in H441 lung cancer cells. An shRNA molecule that did not knockdown RASSF2 was used as a control. Knockdown of RASSF2 expression in the H441 cells was validated by Western Blotting using our RASSF2 antibody (Figure 2(a)). Analysis of cell proliferation confirmed that the RASSF2 knockdown cells exhibited statistically significant ($P < 0.05$) enhanced proliferation compared to control cells (Figure 2(b)).

3.3. Loss of RASSF2 Expression Promotes the Transformed Phenotype. To determine the effects of loss of RASSF2 expression on the transformed phenotype, we plated the H441 RASSF2 knockdown cells in soft agar and compared their ability to form colonies with that of the control cells (Figure 2(c)). The plates were examined 2 weeks after plating and scored for the number of colonies. The cells in which RASSF2 had been knocked down formed significantly more colonies than the control cells ($P < 0.05$) and the colonies that formed were also much larger (Figure 2(c)). These results are consistent with previous reports showing that inactivation of RASSF2 enhances K-Ras-induced cell transformation [24].

3.4. Inactivation of RASSF2 Results in a More Aggressive Phenotype. Overexpression of RASSF2 has been shown to induce cell morphological changes [24], and we have confirmed this in our RASSF2 knockdown cells. Loss of RASSF2 expression resulted in a dramatic alteration in cell morphology. The control H441 cells had a flattened morphology and grew in a monolayer, whereas the cells stably expressing the RASSF2 shRNA constructs became more rounded, piled up on each other, and were more refractile, consistent with a more aggressive and transformed phenotype (Figure 3).

The RASSF2 knockdown cells also exhibited a significant decrease in the degree of adhesion compared to the control cells (Figure 4(a)), a characteristic frequently associated with transformed cells that correlates with enhanced motility. In addition, loss of RASSF2 expression enhanced invasion of the cells. Significantly more cells stably expressing the RASSF2 shRNA constructs were able to invade through a collagen matrix compared to control cells (Figure 4(b)). This result is in agreement with other published reports showing that over expression of RASSF2 inhibits migration [23]. Taken together, these data suggests that loss of RASSF2 expression confers a more aggressive phenotype to lung cancer cells.

3.5. Loss of RASSF2 Expression Activates Growth Promoting Pathways. Since loss of RASSF2 expression resulted in enhanced growth and transformation, we reasoned that inactivation of RASSF2 activated growth promoting pathways. In an effort to determine which prosurvival pathways were activated in the H441 cells knocked down for RASSF2, we analyzed the phosphorylation status of AKT in these cells. Western Blot analysis showed that levels of phosphorylated AKT increased in the cells stably expressing the RASSF2 shRNA constructs relative to control cells (Figure 5). Previous studies have found an association between the methylation status of RASSF2 and the levels of activated AKT. Oral squamous cell carcinomas in which *RASSF2* is methylated showed higher levels of activated AKT [18]. Taken together, our results and those from previous reports suggest that loss of RASSF2 expression results in activation of growth promoting pathways.

3.6. Loss of RASSF2 Expression Confers Resistance to Chemotherapeutic Agents. To determine whether the more aggressive phenotype of the RASSF2 knockdown cells altered their response to chemotherapeutic agents, we treated the cells with taxol or cisplatin, two drugs commonly used in the treatment of nonsmall cell lung cancer, and measured their effects on cell death. Both taxol and cisplatin resulted in increased cell death in the control cells by approximately 40% and 50%, respectively. However, in the cells stably knocked down for RASSF2, taxol had no effect on cell growth and the cisplatin-induced cell death was somewhat abrogated (Figure 6). Thus, loss of RASSF2 expression confers resistance to taxol and cisplatin.

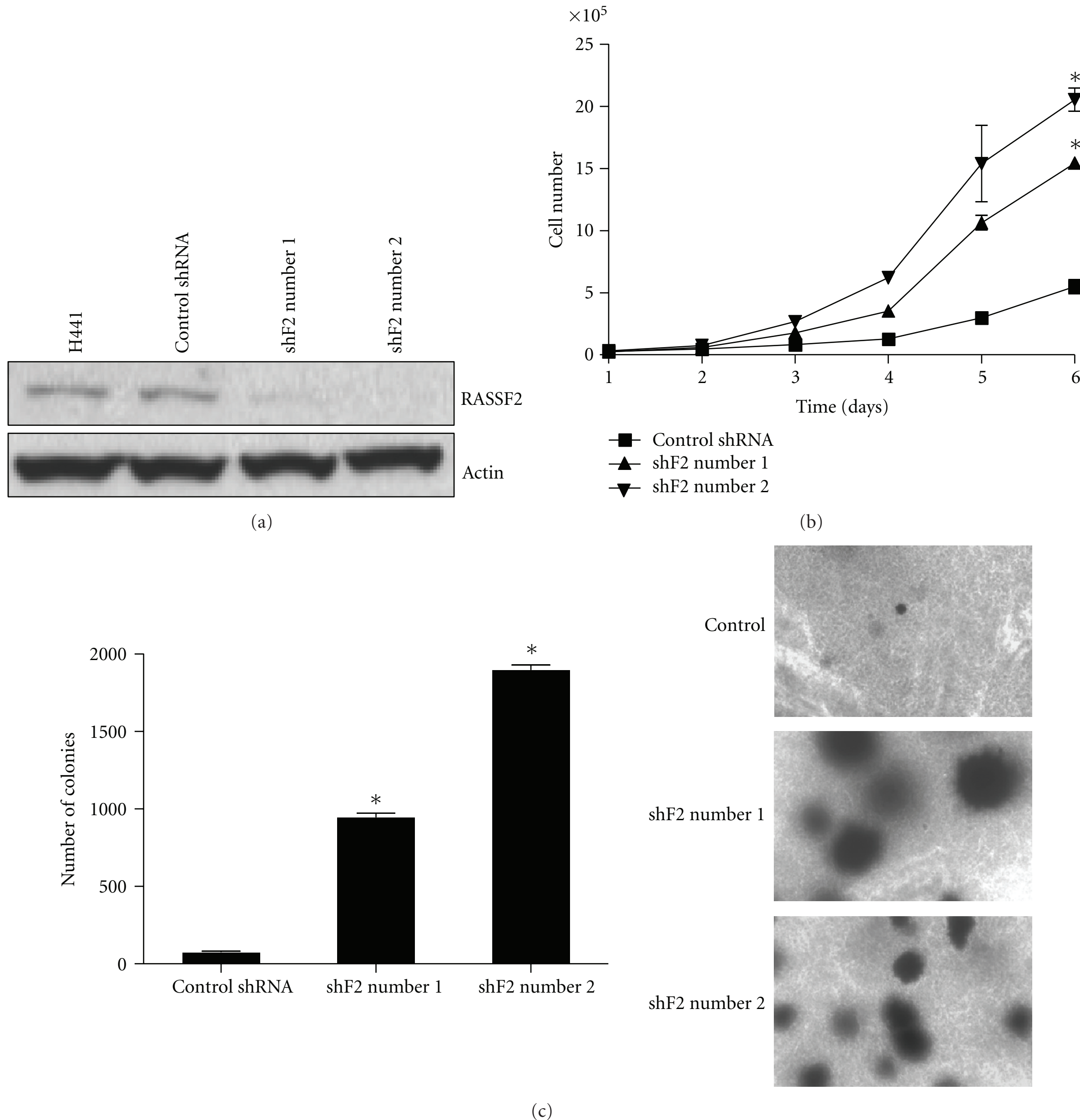
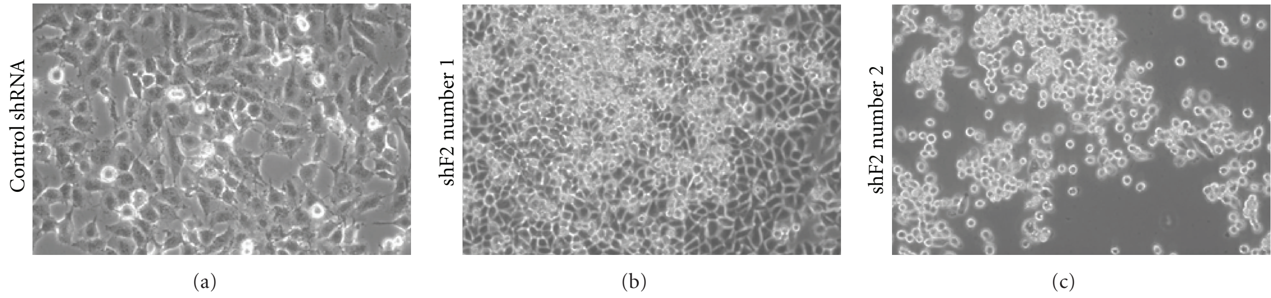


Figure 2: Loss of RASSF2 enhances proliferation and tumorigenicity of lung cancer cells. H441 lung cancer cells were transfected with two independent RASSF2 shRNA constructs and a noneffective shRNA and selected in puromycin for 2 weeks to obtain a population of cells stably expressing the various shRNA constructs. Knockdown of RASSF2 expression was confirmed by Western Blotting (a). Actin was used as a control for protein loading. (b) Growth analysis of the H441 shF2 cells. Cells were harvested and counted at the indicated times to determine cell number. $P < 0.05$ for both shF2-transfected cells compared to control cells. (c) H441 cells stably expressing the shRNA constructs to RASSF2 or control shRNA were plated in soft agar and colony number determined after 14 days. *Statistically different ($P < 0.05$) from cells expressing the control shRNA. The panel on the right shows representative images of the colonies.

4. Discussion

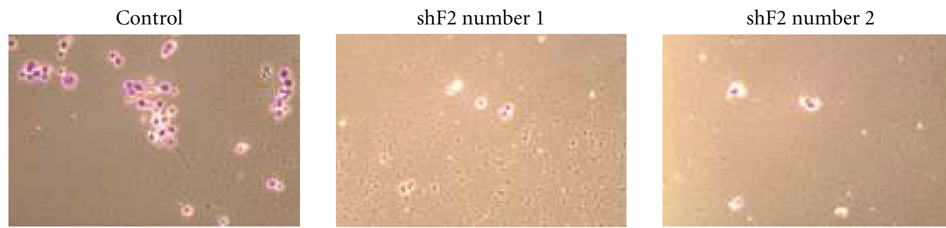
RASSF2 is a novel K-Ras-specific effector that negatively regulates Ras signaling. It has the properties of a tumor suppressor with effects on apoptosis, cell cycle, and cell migration [9, 11, 23, 24]. It may play an important role in tumorigenesis as its expression is silenced in many tumor types by promoter methylation [4, 6, 8, 11–14, 17–19]. Moreover, inactivation of RASSF2 may be an early event in tumorigenesis as it is found inactivated in a high proportion of colon adenomas as well as early stages of prostate cancer [13, 17, 24], raising the possibility that loss of function of RASSF2 may be an initiating event in the development of certain tumor types.

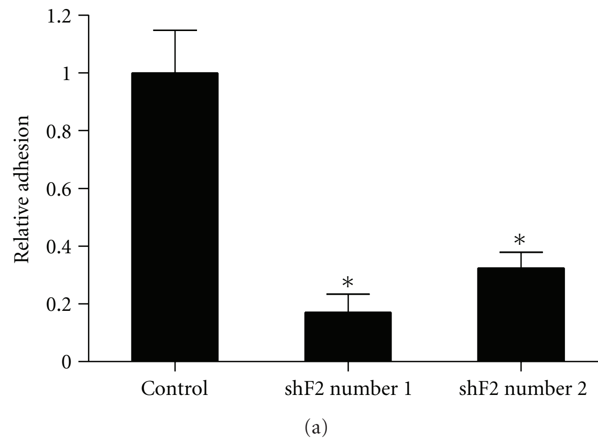
To determine the effects of inactivation of RASSF2 on the transformed phenotype, we established a cell line in which



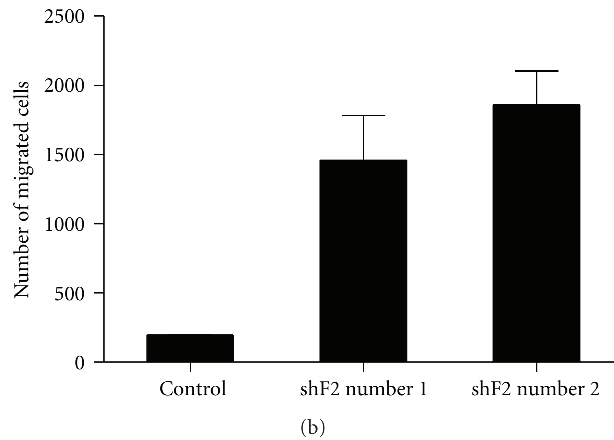
(a) (b) (c)

Figure 3: Inactivation of RASSF2 causes morphology changes. H441 cells stably expressing RASSF2 shRNA constructs and a control shRNA were viewed and photographed using phase contrast at 100x magnification.





(a)



(b)

Figure 4: Loss of RASSF2 decreases cell adhesion and enhances invasion. (a) The H441 control cells and those stably knocked down for RASSF2 were assayed for adhesion as described in Section 2. *Statistically different ($P < 0.05$) from control cells. (b) The cells were assayed for their ability to invade a collagen matrix as described in Section 2. A statistically larger number of cells ($P < 0.05$) that were stably knocked down for RASSF2 were able to migrate through the collagen compared to control cells, indicating that loss of RASSF2 enhances cell invasion.

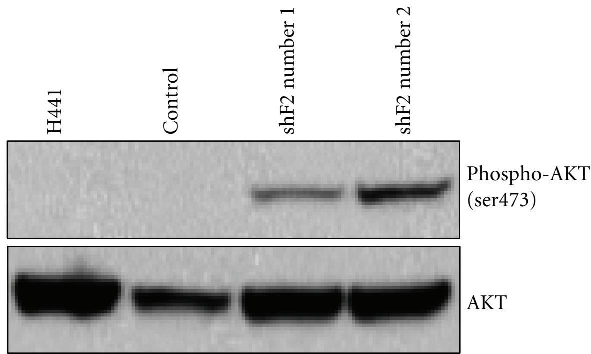


Figure 5: Loss of RASSF2 enhances Ras-mediated signaling pathways. Lysates from the control H441 cells and those stably transfected with the RASSF2 shRNA constructs were prepared, fractionated on SDS gels, and immunoblotted with antibodies against phosphorylated or total AKT. Loss of RASSF2 expression increased the levels of phosphorylated AKT.

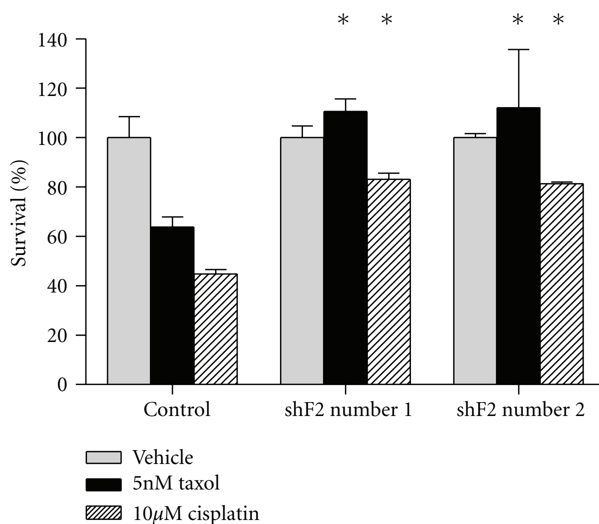


Figure 6: Inactivation of RASSF2 confers resistance to cisplatin and taxol. H441 cells stably transfected with control and RASSF2 shRNA constructs were seeded at 2×10^4 cells per well in 12-well plates and treated with 5 nM taxol or 10 μM cisplatin for 3 days. Cell death was estimated by trypan blue exclusion. Cells stably transfected with the RASSF2 shRNA showed significantly less cell death ($P < 0.05$) compared to the control cells for both taxol and cisplatin treatments.

we stably knocked down RASSF2 expression with RNAi technology. The cells in which RASSF2 had been inactivated adopted a more aggressive phenotype as evidenced by their enhanced growth in traditional 2-dimensional culture as well as their ability to grow in an anchorage independent manner. Consistent with this more transformed phenotype, the RASSF2 knockdown cells were less adherent than control cells, had an altered morphology, and showed an increased invasive potential. These results confirm and support previous studies showing that overexpression of RASSF2 inhibits growth, migration, and transformation [9, 11, 18, 23, 24].

The molecular mechanisms by which RASSF2 inhibits growth are not fully understood. Since RASSF2 has no inherent enzymatic activity or DNA binding properties, it more than likely acts by interacting with other proapoptotic effectors thereby modulating growth inhibitory pathways, much like the better characterized RASSF1A [1, 3]. We have previously shown that RASSF2 forms a direct and physiologically relevant complex with the proapoptotic effector PAR-4 [13], thereby modulating PAR-4 function. Other reports have shown that RASSF2 interacts with the Mst1/2 kinases, thereby regulating the Hippo signaling pathway [14, 15]. Thus, RASSF2 may act as a scaffold integrating multiple tumor suppressor pathways.

There is now conclusive evidence to support RASSF2 as a K-Ras-specific effector. RASSF2 binds to K-Ras in a GTP-dependent manner [11], and our data shows that RASSF2 and K-Ras form an endogenous complex (Figure 1). Furthermore, RASSF2 and K-Ras have been shown to interact at physiologically relevant levels in primary tissue [12]. Moreover, H441 lung cancer cells harbor a mutant K-Ras, and loss of RASSF2 expression in these cells dramatically enhanced their transformed phenotype. This data supports a previous study showing that inactivation of RASSF2 enhances K-Ras-mediated cell transformation in rat kidney cells [24]. It is now becoming clear that oncogenic K-Ras can both promote cellular proliferation as well as stimulate apoptosis [32]. Thus, RASSF2 may serve to keep the growth promoting activity of oncogenic K-Ras in check and loss of RASSF2 expression may then allow the growth promoting effects of activated K-Ras to dominate and override its growth suppressive effects.

In an effort to determine the mechanism behind the aggressive phenotype of the H441 cells in which RASSF2 levels were decreased, we examined the activation status of Ras-controlled signaling pathways and found an increase in activated AKT (Figure 5). This result is consistent with previous studies showing that cell lines in which the *RASSF2* promoter is methylated had higher levels of activated AKT compared to those cell lines in which the RASSF2 promoter was not methylated [18]. Interestingly, no effect on MAPK signaling molecules was observed in cells from *RASSF2* knockout mice during osteoblast differentiation [26]. Thus, it appears that the effects of RASSF2 in modulating Ras-mediated signaling pathways may be somewhat specific. Since RASSF2 can interact directly with activated K-Ras, it remains to be determined exactly how RASSF2 can selectively regulate some Ras-mediated signaling pathways while having little effect on others. RASSF2 interacts preferentially with K-Ras [11] and may thus negatively impact K-Ras-specific signaling pathways without impacting those pathways mediated by H-Ras or N-Ras. It is possible that RASSF2 may have some direct effects on the regulation of AKT activity, but further studies are required to determine whether this is indeed the case.

One possible explanation for the increased growth and transformed phenotype of the RASSF2 knockdown cells is enhanced NF-κB signaling which may be promoted by inactivation of RASSF2. RASSF2 can modulate NF-κB signaling by multiple mechanisms. Firstly, it forms a complex

with IκBα and β [26], thereby directly regulating the NF-κB signaling pathway. Secondly, loss of RASSF2 is associated with elevated levels of activated AKT (Figure 5 and [18]), which can then activate NF-κB signaling. AKT promotes tumor cell invasion which can occur via NF-κB signaling [33–36]. Thirdly, inactivation of PAR-4 results in aberrant NF-κB signaling [37], and we have shown that RASSF2 is required for the full apoptotic effects of PAR-4 [13]. Thus, RASSF2 may regulate NF-κB signaling both directly and indirectly, and loss of RASSF2 expression results in deregulated NF-κB signaling that may be associated with enhanced growth and invasion.

Our data also suggest that loss of RASSF2 expression confers resistance to taxol and cisplatin (Figure 6), 2 frontline therapeutics for the treatment of NSCLC [38]. These two agents offer only a modest improvement in median survival time for patients with advanced NSCLC [38]. Since RASSF2 is inactivated at a high frequency in lung cancer [9, 11, 19] and loss of RASSF2 expression is associated with an increase in activated AKT (Figure 5 and [18], a targeted therapeutic approach using agents against AKT, perhaps in combination with cytotoxic therapy, may prove more successful in at least a subset of lung cancer patients. Currently, there are a number of AKT inhibitors available, some of which are already in clinical trials [39].

In summary, we found that loss of RASSF2 expression enhances the transformed phenotype of lung cancer cells expressing oncogenic K-Ras. This more aggressive phenotype is associated with an increase in activated AKT, suggesting that RASSF2 can negatively regulate Ras-controlled growth promoting pathways. Inactivation of RASSF2 also confers resistance to cisplatin and taxol, suggesting that RASSF2, or the signaling pathways that it regulates, may serve as a target for therapy for lung cancer.

Acknowledgment

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Review Article

RASSF Signalling and DNA Damage: Monitoring the Integrity of the Genome?

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The RASSF family of proteins has been extensively studied in terms of their genetics, structure and function. One of the functions that has been increasingly studied is the role of the RASSF proteins in the DNA damage response. Surprisingly, this research, which encompasses both the classical and N-terminal RASSF proteins, has revealed an involvement of the RASSFs in oncogenic pathways as well as the more familiar tumour suppressor pathways usually associated with the RASSF family members. The most studied protein with respect to DNA damage is RASSF1A, which has been shown, not only to be activated by ATM, a major regulator of the DNA damage response, but also to bind to and activate a number of different pathways which all lead to and feedback from the guardian of the genome, p53. In this review we discuss the latest research linking the RASSF proteins to DNA damage signalling and maintenance of genomic integrity and look at how this knowledge is being utilised in the clinic to enhance the effectiveness of traditional cancer therapies such as radiotherapy.

1. Introduction

RASSF proteins were originally designated on the basis of sequence homology to domains that associate with Ras-like small GTP-binding proteins. These domains are known as Ras association (RA) domains [RalGDS (Ral guanine nucleotide dissociation stimulator)/AF6 (ALL-1 fusion partner from chromosome 6)] and are distinct from Ras-binding domains (RBD) which bind an alternative set of Ras effectors [1, 2]. Ras belongs to a family of small G-proteins that are ubiquitously expressed and oscillate between an inactive, GDP-bound state, and an active, GTP-bound state, in response to diverse cellular signals. Various GTP-bound Ras-like proteins bind effector proteins to mediate distinct biological responses. There are 150 Ras-like proteins encoded in the human genome which can be grouped by homology or functionality, as being similar to Ras, Rho, Rab, Arf (ADP-ribosylation factor), or Ran. While originally suggested to associate with Ras [3], the RASSF family has a differential affinity for Ras-like GTPases, with NORE1 (RAPL/RASSF5) displaying a much greater affinity for the closely related Ras homolog, Rap1B, than H-Ras itself [4]. The RA domain of RASSF1 associates with K-Ras, rather than H-Ras or N-Ras and is also described to associate with Ran [5, 6]. There are now 10 members in the RASSF family (RASSF1-10) subdivided into two distinct subgroups, the classical RASSF proteins (RASSF1-6) and the N-terminal RASSF proteins (RASSF7-10) based on the location of the RA domain [7]. Little is known about the GTP-binding proteins that may interact with the majority of the RASSF family or how they function but the potential exists for a greater number of signalling connections. In addition to an RA domain, the classical RASSF proteins also have a protein-protein interaction motif known as the SARAH domain that is responsible for scaffolding and regulatory interactions [8]. This domain is a short coiled-coil region and so named due to its location in the extreme C-terminus of genetically linked *Drosophila* proteins; Salvador (hSav1/WW45), dRASSF and Hippo (hMST1/2) (SARAH: SAlvador, RAssf, Hippo) which can form both homo- and heterodimers [9]. The N-terminal RASSFs lack an identifiable SARAH domain, although the SMART database predicts that RASSF7, 8 and 10 contain extensive coiled-coil regions, which can dimerise [10].

RASSF1A and RASSF5A [also known as NORE1A (Novel Ras Effector 1 isoform A)] also contain an N-terminal atypical diacylglycerol/phorbol ester-binding (DAG) domain also

known as the protein kinase C conserved region (C1) domain that contains a central zinc finger (Zinc-binding domain) [11]. The Zinc finger in the RASSF family members is denoted "atypical" because it lacks critical residues required for binding of phorbol esters or DNA and therefore probably mediates protein-protein interactions. Indeed, structural analysis indicates that the C1 domain of NORE1A associates with the RA domain to occlude RAS association [12]. As none of the family members have any known enzymatic activity they are thought to be scaffold/adaptor proteins using these binding domains to bring target proteins together to impart their functions.

There are a number of reviews that introduce the RASSF family and the pathways within which they function; however, this paper will focus on the emerging roles of the RASSF family and their effectors in the response to DNA damage. The best described protein in this family with respect to DNA damage is RASSF1 thus the review will concentrate on this protein with particular reference to a recently elucidated signalling network from RASSF1A and the potential clinical significance of targeting this pathway [13].

2. RASSF1

It had long been suspected that the 3p21.3 region of the human genome harboured one or more important tumour suppressors because loss of heterozygosity (LOH) was found at this locus in lung, breast, and kidney tumours and genetic instability in this region is the earliest most frequently detected deficiency in lung cancers [14–20]. This 120 kb region contains 8 genes namely *CACNA2D2, PL6, 101F6, NPRL2/G21, ZMYND10/BLU, RASSF1/123F2, FUS1,* and *MYAL2*. However, none of these candidate genes are frequently mutated in cancers [16, 21]. At the same time as these LOH studies, Dammann et al. identified RASSF1 as an interacting partner of the DNA damage repair protein xeroderma pigmentosum complementation group A (XPA) [22]. While the role of RASSF1 in nucleotide excision repair could not be verified, it may yet prove to be significant given the emerging role of RASSF1 in the DNA damage response. The *RASSF1* gene consists of 8 exons spanning a region of about 11 kb. The C-terminal showed high-sequence homology with NORE1, containing an RA domain and thus the gene was named *RASSF1* for Ras association domain family member 1 [22]. Alternative splicing generates 8 isoforms A–H from promoters held within 2 CpG islands. The first CpG island encompasses the promoter regions for RASSF1A, D, E, F, and G. Epigenetic inactivation by DNA methylation at this CpG island is one of the most common events in human cancers (reviewed in [23–25]). This methylation has recently been attributed to HOXB3 driven overexpression of the DNA methyltransferase, *DNMT3B* [26]. RASSF1B, C, and H are generated from a promoter located within the larger 3′ CpG island [27]. This commonly remains unmethylated in cancers and consequently cells retain expression of these isoforms [23]. RASSF1A and RASSF1C are the major transcripts of the *RASSF1* gene and are expressed ubiquitously in normal tissues [28].

3. RASSF1A

Exogenous expression of RASSF1A reduces colony formation in soft agar and reduced tumourigenicity in nude mice [22, 29–31]. Similarly, reexpression of RASSF1A using demethyltransferase inhibitors such as zebularine and 5-aza-2′-deoxycytidine caused significant growth arrest in ovarian cancer cell lines [32]. Reciprocally, RASSF1A knockout mice develop spontaneous tumours, particularly when combined with a knockout of p53, highlighting the significance of RASSF1A in tumour development [33–35]. In addition these RASSF1A$^{-/-}$, p53$^{-/-}$ mice showed high levels of aneuploidy/tetraploidy suggesting an important role for RASSF1A in maintaining genomic integrity. RASSF1A has been shown to have many roles in cell cycle control and microtubule organisation [23, 27], the response to DNA damage is, however, only beginning to be elucidated. It is therefore timely to present these pathways and highlight their importance to the DNA damage response, genomic integrity, and cell survival during cancer development.

4. RASSF1 Phosphorylation

The majority of the phosphorylation of RASSF1A has being attributed to the phosphorylation of Serine 202/203. These sites have been demonstrated to be targeted by a number of kinases including, both CDK (Cyclin-Dependent Kinase) and Aurora kinases [36–40]. These phosphorylation events prevent the association of RASSF1A with microtubules during prometaphase. The phosphorylation of RASSF1A on these sites also coordinates the regulation of mitosis by controlling activation of the anaphase-promoting complex/cyclosome (APC/C), and regulation of syntaxin16 to promote cytokinesis [39, 40]. Loss of phosphorylation at these sites leads to defects in mitosis resulting in aneuploidy and genomic instability.

In the DNA damage response phosphorylation of RASSF1A serine 131 (S131) is emerging as an important phosphorylation site. The initial kinases that respond to breaks in DNA are the phosphatidyl-inositol 3-kinase like kinases ATM (Ataxia Telangiectasia Mutated), ATR (ATM- and Rad3-Related), and DNA-PK$_{cs}$ (DNA-dependent protein kinase catalytic subunit) [41]. RASSF1A has a consensus site for ATM phosphorylation on serine 131 that is conserved in vertebrates and unique amongst family members and has recently been confirmed as a bone-fide target for ATM [13, 42]. Serine 131 phosphorylation appears important for RASSF1A activation and inactivating mutations of this site have been identified in human cancers [43]. Indeed Shivakumar et al., showed that mutation of the predicted phosphorylation site, S131F, removed the ability to induce cell cycle arrest and block cell proliferation [43]. ATM-dependent phosphorylation at the 131 site is also restricted by S131F and disables the ability of RASSF1A to respond to various DNA-damaging agents [13]. Mutations near the ATM site are hypothesised to function by inactivating ATM phosphorylation. One of these is a nonsynonymous single nucleotide polymorphism (SNP) at p.RASSF1A-A133S (rs2073498),

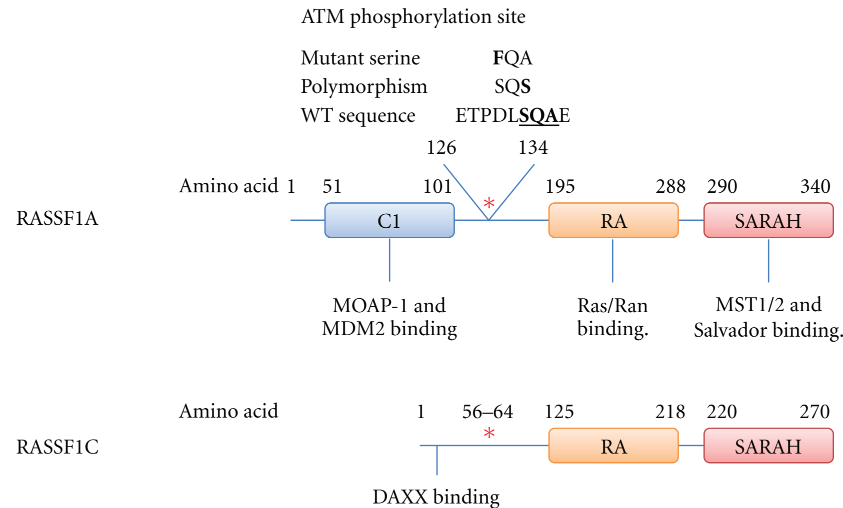

Figure 1: Cartoon depicting the interactions of RASSF1A and RASSF1C. RASSF1A and RASSF1C share a common C-terminal aminoacid sequence, which includes the ATM phosphorylation site (red asterisk), the RA domain, and the SARAH domain but differs at the N-terminal. RASSF1A has a C1 domain which interacts with MOAP-1 and MDM2. RASSF1C lacks the C1 domain but has an alternative DAXX interaction domain. Serine 131 of RASSF1A has been shown to be mutated from serine (S) to phenylalanine (F). An alanine (A) to Serine (S) polymorphism also exists at the 133 site.

which has significant allele frequencies in human populations (http://hapmap.ncbi.nlm.nih.gov/). The minor allele of the SNP encodes a serine (A133S) and decreases the ability of RASSF1A to become phosphorylated which, like S131F, results in a defective G1 arrest [43]. This suggests that sequence changes to the ATM consensus sequence (aminoacids 125–138) may severely inhibit the function of RASSF1A by disrupting the phosphorylation of S131 and preventing the activation of RASSF1A (Figure 1).

RASSF1A association with Ran directs the formation of a Ran-GTP gradient between the spindle poles and the metaphase plate which is important for the formation of mitotic spindle and for successful completion of mitosis. RASSF1A targets MST1/2 kinase activity towards the RanGEF (GTP exchange factor) RCC1, which inhibits its function and results in elevated Ran-GTP near the metaphase plate. Taken together, these studies indicate that RASSF1A is important for the maintenance of genomic stability by acting as an integrity checkpoint factor. Loss of RASSF1A is likely to weaken the prometaphase checkpoint and increase the potential to create genomic instability and DNA damage leading to cancer development. Indeed, the restriction of RASSF1A activity by modulation of the ATM site may be linked to numerous observations regarding the early onset of tumours in individuals carrying one minor allele of the p.RASSF1A-A133S polymorphism [44, 45]. This has been controversially linked to the exacerbation of a BRCA1/2 genomic instability phenotype; however, the inconsistency may be due to confounding factors other than BRCA2 and may be due to genomic instability via defects in RASSF1A itself [46]. All this may indicate that DNA damage activation of RASSF1A may provide an extra level of regulatory response, whereby the prometaphase checkpoint senses cells entering into mitosis with DNA damage.

5. Regulation by Domain Interaction

As a scaffold, RASSF1A must exert its tumour suppressor function through its interaction domains. The two most important domains in the context of DNA damage are the C1 domain and the SARAH domain. The most significant binding partners identified to interact with the C1 domain are the TNF-R1/TRAIL-R1—Modulator of Apoptosis-1 (MOAP-1) complexes and the MDM2/DAXX/HAUSP/p53 complex [47, 48] (Figure 1). MOAP-1 and RASSF1A are recruited to either TNF-R1 or TRAIL-R1 in response to TNFα stimulation. RASSF1A binds MOAP-1 causing an activating conformational change to the structure of MOAP-1. The active structure can bind to the proapoptotic Bcl-2 family member BAX which creates a pore in the outer mitochondrial membrane leading to the release of cytochrome C and induction of caspase-dependent apoptotic signalling pathways [47, 49]. BAX and the associated negative regulator BAK tightly regulate the cell's response to apoptotic signals and are often coordinated with other apoptotic signals such as DNA damage. It is reasonable to assume that RASSF1A-MOAP-1 may be affected by DNA damage but whether this contributes to the regulation of BAX/BAK at the mitochondria remains uncertain.

The response of tumour suppressor p53 to DNA damage results in a variety of outcomes including cell cycle arrest, apoptosis, and senescence, combining to protect the integrity of the genome [50, 51]. In unstressed cells p53 levels are low, being controlled by the RING domain-containing E3 ubiquitin ligase MDM2 (Mouse Double Minute 2) [52, 53]. Induction of DNA damage results in phosphorylation of p53 by the DNA damage checkpoint proteins ATM (on serine 15) and CHK2 (Checkpoint Kinase 2) (on serine 20) [54, 55]. These phosphorylation events combine with an ATM-mediated restriction of MDM2 activity to stabilize p53. Song et al. have

recently shown that the C1 domain of RASSF1A can bind and sequester MDM2 in an ATM-dependent manner [48]. They describe a complex consisting of MDM2, DAXX (death-domain-associated protein), and HAUSP1 (a deubiquitinating enzyme). HAUSP1 removes ubiquitin molecules from MDM2 and increases its stability. Upon DNA damage, ATM activates RASSF1A driving its association with MDM2, potentially through phosphorylation at S131. RASSF1A disrupts the MDM2-DAXX-HAUSP1 complex, sequestering MDM2 away from p53, and preventing HAUSP1-regulated deubiquitination of MDM2 promoting its degradation. Release of DAXX from the complex is thought to allow DAXX relocation to the plasma membrane where it can bind the death receptor Fas and activate c-Jun NH_2-terminal kinase (JNK) [56]. Activated p53 exerts its tumour suppressor function by acting as a transcription factor. It has recently been shown that the *RASSF1* promoter is a target for p53 [57]. Interestingly, p53 appears to downregulate the transcription of RASSF1A hinting at a second mechanism through which p53 can negatively regulate itself in addition to the upregulation of MDM2.

RASSF1A makes two significant interactions through its SARAH domain; the first with mammalian sterile 20-like kinases 1 and 2 (MST1/2) and the second to the scaffold protein Salvador (Figure 1). The RASSF1A interaction with MST1/2 leads to an increase in the local concentration of MST molecules allowing them to undergo transphosphorylation and autoactivation [58]. The interaction further stabilises the MST1/2 kinase activity by preventing dephosphorylation of MST1/2 [59]. MST1/2 were initially cloned from lymphoid cDNA library when looking for human relatives of *Saccharomyces cerevisiae* protein Ste20 and subsequently shown to be activated by a wide variety of cellular stresses [60–63]. Of note is that both *Drosophila* dMST (Hippo) and MST2 are activated in response to DNA damage. In mammals, DNA damage induction of MST2 requires direct binding of RASSF1A- and ATM-mediated phosphorylation of S131 [13, 64, 65]. Interestingly MST1 was shown to be able to activate p53 in response to cisplatin-induced DNA damage by phosphorylating and inactivating Sirt1, a deacetylase that inactivates p53 [66]. Additional substrates of MST kinases that may prove subject to DNA damage are the histones H2B and H2AX, JNK and FOXO transcription factors [67–70]. However, a clear example of signalling through RASSF1A-MST after DNA damage is the recruitment and activation of the large tumour suppressor kinases 1 and 2 (LATS1/2) [71, 72].

Studies on the *Drosophila* homolog of MST1/2, Hippo have discovered that the pathway through Warts (LATS1) is responsible for controlling proliferation and apoptosis and is conserved in both vertebrates and invertebrates. Mutations in pathway member's Hippo (MST1/2), Warts (LATS1/2), Salvador (WW45), or Mats (Mob1 as a tumour suppressor) result in vast tissue overgrowth. The pathway generates a signal to inhibit Yorkie (YAP). Yorkie mutants therefore inevitably show a reduced tissue growth phenotype (reviewed in [73]). Yorkie is a non-DNA binding transcriptional coactivator that binds Scalloped (TEAD1-4) leading to the upregulation of proteins such as Cyclin E and Diap-1 to promote cell division and inhibit apoptosis. In this case Warts phosphorylates Yorkie creating a site for 14-3-3 binding. This sequesters Yorkie in the cytoplasm inhibiting its oncogenic activity [74]. In mammals, in the presence of RASSF1A and a DNA damage signal, LATS1 phosphorylation of YAP maintains a pool of YAP in the nucleus which switches binding partner from the antiapoptotic, YAP-TEAD complex to a proapoptotic YAP-p73 complex [75]. The interaction between YAP1 and p73 stabilises p73 by preventing its nuclear export and subsequent degradation [76–78]. YAP1 functions as a coactivator of p73 and this complex upregulates p73 responsive genes such as the proapoptotic BH3 only Bcl-2 family member, PUMA [79, 80]. This idea is in agreement with the finding that both LATS1 and LATS2 mediate apoptosis through p53. In certain cases LATS2-mediated apoptosis is p53 independent, potentially indicating a switch to YAP1 and p73 [13, 81, 82].

LATS2 has been shown to activate p53 both directly, by binding to and inhibiting MDM2 and indirectly by driving the nuclear accumulation of ASPP1 (apoptosis-stimulating protein of p53) [83, 84]. Interestingly, cytoplasmic ASPP1 appears to behave in an opposite manner and inactivates the ability of LATS1 to interact with YAP1 [85]. As RASSF1A activates LATS1/2 in response to DNA damage this could potentially drive ASPP1 activation of p53 and contribute to the overall p53 response. Interestingly the *Drosophila* ASPP protein (dASPP) has also been shown to interact with dRASSF8 to regulate C-terminal Src kinase (dCsk) and adherens junctions [86], a site key to the regulation of the core hippo pathway [87].

LATS2 has been implicated in the G1 tetraploidy checkpoint, a process that is thought to be driven by LATS2 activation by ATR and leads to direct stabilisation of p53 [83, 88]. Active p53 then creates a positive feedback loop with LATS2 by upregulating its activity further [88]. In response to UV radiation CHK1 activation by ATR has been shown to activate LATS2 [89].

Although not addressed in a RASSF1A-dependent manner, YAP forms an additional DNA damage promoted complex with the transcription factor early growth response 1 (EGR1) [90]. The interaction promotes enhanced transcriptional activity of EGR1 for the Bcl-2-associated X (BAX) promoter. Thus YAP can act as an oncogene and a tumour suppressor in a RASSF1A-context-dependent manner.

In *Drosophila* dRASSF and Salvador are known to com pete for MST binding. Here Salvador acts as an adaptor to bring Hippo and Warts together to activate the hippo pathway, which is antagonised by dRASSF [91]. In mammals, however, RASSF1A can bind both MST1/2 and Salvador at the same time using different regions with the SARAH domain. Using an L308P mutant of RASSF1A that cannot bind MST but remains bound to Salvador, Donninger et al. have shown that the RASSF1A Salvador interaction can activate p73 in an MST-independent manner [92].

6. RASSF1C

RASSF1C is the second ubiquitously expressed isoform of the *RASSF1* gene. Like RASSF1A, RASSF1C contains the ATM

consensus sequence (Figure 1). This site, at Serine 61, has not yet been confirmed but the sequence is identical between RASSF1A and RASSF1C at this site so it is plausible to suggest that RASSF1C is also phosphorylated and activated by ATM. Indeed, the Serine 61 to phenylalanine (S61F) mutant of RASSF1C was unable to block the genomic destabilising effects of Ras which can be ablated by overexpression of wild-type RASSF1C in the embryonic kidney cell line 293T and human lung tumour cell line NCI-H1299 [93]. This suggests that DNA damage activation of RASSF1C may require phosphorylation of Serine 61 (RASSF1A-131) site. Further to this, RASSF1C has recently been implicated in a DNA damage response pathway involving DAXX (which also binds to RASSF1A) and JNK [94] (Figure 1). In unstressed conditions RASSF1C is shown to be in a complex with DAXX in the nucleus, recently resolved by NMR [95]. Upon ultraviolet radiation or MMS-induced DNA damage this interaction is lost allowing RASSF1C to move to the cytoplasm where it aids the activation of SAPK/JNK signalling [94]. DAXX, however, remains in the nucleus concentrating at PML bodies. The signal that leads to release of RASSF1C from DAXX is unknown; however, it would be interesting to see if the signal relies upon the ATM phosphorylation site. Conversely, another study has identified that RASSF1C, far from being activated by DNA damage, is targeted for degradation under stress conditions. Exposure to UV radiation or treatment of cells with doxorubicin leads to RASSF1C phosphorylation by GSK3β creating a phosphodegron at S19/23 which is bound to by SCF$^{\beta\text{-TrCP}}$ targeting RASSF1C for degradation [96]. This GSK3β-dependent degradation was shown to be inhibited by the PI3-K/AKT pathway. Since AKT activity can lead to RASSF1C upregulation it suggests that RASSF1C could function as an oncogene. This is in keeping with several recent reports showing that RASSF1C increased cell proliferation in lung cancer cells and migration in breast cancer cell lines [97, 98].

7. Therapeutic Implications of RASSF1A Loss

One of the most common and widespread events to occur during cancer development is the loss of RASSF1A expression. This loss is due to methylation of the upstream CpG islands in the *RASSF1* gene [22, 29]. The frequency of epigenetically driven loss of RASSF1A correlates well with the increasing grade of the tumour. Methylation has been reported in over 37 tumour types (comprehensively reviewed in [24, 99]) and is thought to be an early event in breast and thyroid tumourigenesis, childhood neoplasia, and endometrial carcinogenesis [27].

RASSF1A methylation correlates with a decreased responsiveness to DNA-damaging therapies [100–102]. The DNA methyltransferase (DNMT) inhibitor zebularine has been used to effectively reexpress RASSF1A and show an increase in cancer cell sensitivity to radiation-induced damage *in vitro* and *in vivo* [101] as well as to cisplatin [32]. Dote et al. showed that 48 h treatment with zebularine, which corresponded to the maximum reexpression of RASSF1A increased the radiosensitivity of PaCa, DU145, and U251 cancer cell lines by 1.5 times and caused an increased tumour delay in U251 xenograph models in mice [101]. A 48 h treatment with zebularine also increased cancer cell sensitivity to DNA damage and a 16-fold reduction in IC_{50} of cisplatin in resistant ovarian cancer cell lines [32]. Sensitivity of testicular germ cell tumours to cisplatin could also be enhanced by another DNMT inhibitor that is in clinical trials, 5-aza-2′-deoxycytidine [103]. Interestingly, they noted that effectiveness of the 5-aza-2′-deoxycytidine treatment was dependent on the level of DNMT3B levels. The higher the DNMT3B level the greater the effect. The most significant target gene for DNMT3B was shown to be RASSF1A (as mentioned above) and thus it can be extrapolated that the increase in sensitivity to cisplatin is due to the reexpression of RASSF1A. Reexpression of RASSF1A using 5-aza-2′-deoxycytidine or reintroduction of RASSF1A into the hepatocellular carcinoma cell line, SMMC-7721, was also shown to increase sensitivity to chemotherapeutics such as fluorouracil, mitomycin, and cisplatin [104]. Together these results support a clinically relevant role for RASSF1A in the DNA damage response that is backed up by phase I and II clinical trials in myelodysplasia and leukaemia patients where 5-aza-2′-deoxycytidine has shown efficacy both alone and in combination with the histone deacetylase (HDAC) inhibitor valproic acid [105, 106]. Therapeutic failure upon RASSF1A loss can also be counteracted by targeting the downstream DNA damage responsive signalling pathway. Direct activation of BAX via the BH3 mimetic ABT-737 has recently put forward as a potential treatment for RASSF1A methylated medulloblastoma [107]. The role of RASSF1A in checkpoint activation and maintenance of genomic integrity is highlighted in a study by Zhang et al. which showed a significant increase in DNA damage caused by aflatoxin B_1 in tumour tissues where RASSF1A has been lost due to DNA methylation [108].

8. Other RASSFs and DNA Damage

This paper has concentrated primarily upon the role of RASSF1 in DNA damage; however, it is worth noting that other RASSF proteins have also been linked to DNA damage pathways. The RASSF2 gene resides on chromosome 20. The gene can be spliced into two very similar proteins RASSF2A and RASSF2C both of which contain the RA domain and the SARAH domain. They show 28% identity to RASSF1A and like RASSF1A, the promoter has been shown to be inactivated by hypermethylation in primary tumours [109–114]. RASSF2 has been reported to be upregulated in lymphocytes from individuals exposed to ionising radiation [115]. RASSF2 has also been shown to associate with, and is phosphorylated by, MST2 leading to stabilisation of MST2 and the generation of proapoptotic signals [116].

The *RASSF6* gene is located on chromosome 4. While the expression of RASSF6 is lost in cancer, *in silico* analysis did not find any CpG islands located near the promoter; therefore, it is assumed that this loss is not due to DNA methylation [117, 118]. RASSF6 is known to activate apoptosis in both caspase-dependent and -independent mechanisms in response to TNFα; however, it is unknown whether it is also

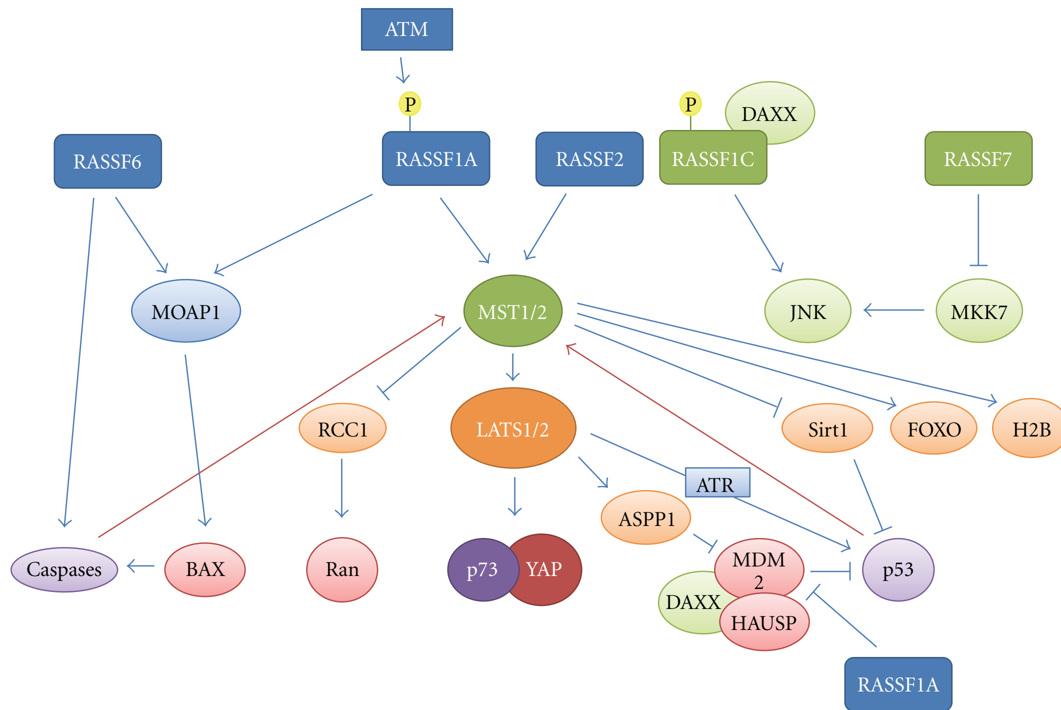

Figure 2: Cartoon depicting DNA damage activated pathways downstream of RASSF family members. RASSF family members, activated by DNA damage, signal through various intermediates (primary interaction: light blue and green [involving RASSF1C or RASSF7]; secondary: orange and tertiary: red) to activate p53, p73, and caspases (purple) to control apoptosis, genome stability, and senescence. Feedback loops exist from caspases and p53 that further activate the pathways and amplify the signal. RASSF1A can also directly sequester MDM2 leading to p53 activation. RASSF1C can transfer DNA damage signals from the nucleus to the cytoplasm by activating JNK signalling. RASSF7 acts as an oncogene inhibiting the activation of JNK.

activated by DNA damage signals [118]. RASSF6 contains both the RA domain and SARAH domain and like RASSF1A it has been shown to bind to MOAP-1 [117], which could be responsible for its induction of apoptosis in response to TNFα. Unlike other family members, RASSF6 contains a number of ATM consensus sites (SQ/TQ) upstream of the RA domain; however it is not clear if these are functional.

RASSF family members efficiently form heterodimers [119]. This provides a potential mechanism through which additional RASSF proteins could be involved in DNA damage signalling. A heterodimer between RASSF1A and RASSF5A has been suggested to be important for the interaction of RASSF1A with Ras [120]. Given that each of the RASSF proteins above is thought to impart its tumour suppressor function through the MST kinases we could propose that heterodimeric interactions between RASSF family members may be important for their DNA damage-induced apoptotic signalling.

RASSF7 is the best studied N-terminal RASSF protein and the first to be shown to be linked to the DNA damage response. Located on chromosome 11 close to the H-Ras gene (*HRAS1*), it forms part of a microsatellite that is associated with increased cancer risk [121–123]. Unlike the majority of the RASSF family members that are silenced in cancer, RASSF7 has been shown to be upregulated in a number of cancers including pancreatic, endometrial, and ovarian [124–128]. The upregulation of RASSF7 in cancers suggests an oncogenic function, the mechanism of which has only just started to be explored. RASSF7, in concert with N-Ras, is thought to suppress the activation of JNK in response to low doses of UV radiation by binding and inhibiting MKK7, preventing its interaction with JNK. At higher doses of UV, RASSF7, like RASSF1C, is targeted for degradation through an ubiquitin-dependent mechanism. This frees MKK7 to activate a stress response through JNK [129].

9. Conclusion

Ras-association domain containing family members are important tumour suppressors involved in linking cellular stresses to cell cycle arrest and apoptosis (Figure 2). RASSF1A is an adaptor protein with three major interaction domains through which it imparts its functions. Each of these domains is involved in binding different effector proteins in response to DNA damage. The C1 domain binds MDM2 to stabilise p53 and the RA and SARAH domains are required to activate the mammalian Hippo pathway. The mammalian homolog of Hippo, MST1/2, can activate apoptosis in response to cellular stresses either directly, in the case of FOXO1 and histone H2B or via LATS1/2. RASSF2, RASSF5, and RASSF6 which share the RA and SARAH domains with RASSF1A have also been shown to active MST1/2 to induce

apoptosis as well as being able to induce apoptosis independently of the Hippo pathway. LATS1 and 2 have been implicated in apoptosis by stabilising both p53, either directly through an interaction with MDM2 or indirectly via ASPP1 and stabilising p73 via YAP, in response to DNA damage. RASSF1C has been shown to be released from DAXX and p53 upon DNA damage where it can go and transmit the damage signal from the nucleus to the cytoplasm by activating JNK signalling. Each of these proteins appears to act both upstream and downstream of the "guardian of the genome" p53 to create a network which feeds back upon itself to enhance the DNA damage signaling within the cell. Greater than 50% of human tumours has either lost or mutated p53. Disruption of these networks will inactivate p53 and may contribute to tumourigenesis in a number of the cases where wild-type p53 is retained. Although not correlated with p53 loss or mutation, RASSF proteins are epigenetically lost in human cancers by DNA methylation. It has been shown that, as with p53, loss of *RASSF1* expression is associated with more aggressive tumours and increased resistance to radiation-induced DNA damage and platinum-based drugs. DMNT inhibitors such as zebularine have been shown to reexpress RASSF1A and increase the radiosensitivity of these cancers suggesting that reexpression of RASSF1A and other silenced RASSFs maybe a path through which chemoradioresistant tumours can be combated.

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Review Article

RASSF1A Signaling in the Heart: Novel Functions beyond Tumor Suppression

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The RASSF proteins are a family of polypeptides, each containing a conserved Ras association domain, suggesting that these scaffold proteins may be effectors of activated Ras or Ras-related small GTPases. RASSF proteins are characterized by their ability to inhibit cell growth and proliferation while promoting cell death. RASSF1 isoform A is an established tumor suppressor and is frequently silenced in a variety of tumors and human cancer cell lines. However, our understanding of its function in terminally differentiated cell types, such as cardiac myocytes, is relatively nascent. Herein, we review the role of RASSF1A in cardiac physiology and disease and highlight signaling pathways that mediate its function.

1. Introduction

The Ras association domain family (RASSF) consists of 10 members: RASSF1-10. Additionally, splice variants of RASSF1, 5 and 6 have been identified [1]. Importantly, all isoforms contain a Ras association (RA) domain either in their C-terminal (RASSF1-6) or N-terminal (RASSF7-10) regions [2]. To date, no known catalytic activity has been described for this family, and the general consensus supposes that RASSF proteins function as scaffolds to localize signaling in the cell. Accordingly, protein-protein interactions are critical in mediating their biological functions. RASSF1 isoform A (RASSF1A) is the most characterized member of the RASSF family. This paper will focus primarily on RASSF1A and its role in cardiovascular biology.

2. RASSF1A

RASSF1A was first identified and described by Dammann et al. in 2000 [3]. The *RASSF1* gene encodes multiple splice variants, including the two predominant isoforms, RASSF1A and C. The RASSF1A isoform is the longest variant of the *RASSF1* gene. Structurally, RASSF1A is a product of exons 1α, $2\alpha/\beta$, 3, 4, 5, and 6, while RASSF1C consists of exons 2γ, 3, 4, 5, and 6. Both isoforms contain a C-terminal RA domain; however, RASSF1A has an additional C1 domain that is not present in RASSF1C.

The *RASSF1* gene is located on Chr3p21.3 [3]. This short arm of chromosome 3 is known to exhibit loss of heterozygosity in many tumor models and is thought to harbor tumor suppressor genes. As the literature has shown, RASSF1A fits this description. The *RASSF1A* promoter contains a CpG island that shows a high frequency of hypermethylation in tumors, thereby silencing RASSF1A expression in many human cancers including lung, breast, ovarian, renal, and bladder [4–7]. RASSF1A expression is also lost in numerous cancer cell lines, while RASSF1C expression is seemingly unaffected [4]. Interestingly, recent work suggests that RASSF1C may actually promote tumor progression [8, 9], further distinguishing these two splice variants.

All RASSF proteins have an RA domain, which is thought to necessitate their binding to activated, GTP-bound Ras proteins. While RASSF5 (Nore1) is thought to bind Ras

directly, whether RASSF1A is able to associate with Ras is less clear. It has been shown that RASSF1A binds K-Ras *in vitro* [10], and an interaction between ectopically expressed RASSF1A and activated K-Ras has been observed in HEK293 cells [11, 12]. However, other work has found that this interaction only occurs in the presence of Nore1, arguing for an indirect association [13]. Importantly, to our knowledge, there are no reports demonstrating the interaction of endogenous RASSF1A and Ras proteins.

RASSF1A has several key biological functions typical of tumor suppressor proteins. It has been implicated in the negative regulation of cell cycle progression, cell proliferation, and cell survival [2]. RASSF1A has been shown to localize to microtubules of proliferating cells, increasing microtubule stability and inhibiting cell division [14, 15]. This may be mediated through direct binding or though interaction with microtubule-associated proteins such as C19ORF5 [16]. RASSF1A has also been shown to inhibit proliferation by inhibiting the accumulation of cyclin D1 and arresting cell division [17, 18].

RASSF1A also promotes apoptosis, which can reportedly occur through multiple mechanisms and is likely cell-type dependent. One mechanism that mediates the apoptotic function of RASSF1A involves protein interaction with modulator of apoptosis-1 (MOAP-1 or MAP-1) [19]. MOAP-1 is normally sequestered in an inactive form in healthy cells. Upon death receptor stimulation, RASSF1A binds MOAP-1, causing its activation and subsequent association with Bax, which leads to apoptosis [19]. Previous work has also demonstrated enhancement of RASSF1A/Mst-mediated cell death by the scaffold CNK1 [20].

2.1. RASSF1A and Hippo Signaling. RASSF1A can also elicit inhibitory effects on growth and survival through engagement of the Hippo pathway. The Hippo signaling pathway is a highly conserved kinase cascade that was originally discovered in Drosophila and has been shown to be a critical regulator of cell proliferation, survival, and organ growth [21]. Three members of this pathway, dRASSF, Salvador and Hippo, contain the SARAH (Salvador-RASSF-Hippo) domain, which is conserved in its mammalian counterparts RASSF1-6, WW45, and Mst1/2, respectively [22]. The SARAH domain is critical for homo- and heterodimerization between components [23–27]. While the Drosophila ortholog dRASSF is known to antagonize Hippo activation in the fly [28], it has been demonstrated that RASSF1A promotes phosphorylation and activation of Mst 1/2 by inhibiting the phosphatase PP2A in mammalian systems [29, 30].

The biological relevance of RASSF1A-mediated activation of Hippo signaling has also been investigated. Matallanas et al. reported a RASSF1A-Mst2-Yap-p73-PUMA signaling axis that promotes apoptosis in mammalian cells [31]. Hippo signaling is also important for maintaining intestinal homeostasis and tissue regeneration in response to injury. Mouse models with conditional disruption of either Mst1/2 or Sav1 in the intestinal epithelium displayed hyperactivation of Yes-associated protein (Yap), increased intestinal stem cell (ISC) proliferation, and increased polyp formation following dextran sodium sulfate (DSS) treatment [32, 33]. Similarly, loss-of-function mutations of Hippo components in the fly midgut caused increased ISC proliferation [34]. These findings suggest that perhaps Hippo signaling serves a more global role in regulating organ integrity, structure, and response to injury, and that perturbation of this pathway can lead to aberrant growth and dysfunction.

3. Cardiovascular Function of RASSF1A

In 2005, two independent groups generated and published findings regarding the systemic deletion of the *Rassf1a* gene variant in mice [35, 36]. Both described similar phenotypes involving the spontaneous generation of tumors, particularly in aged mice, thus further supporting the notion that RASSF1A is a bona fide tumor suppressor [35, 36]. Not surprisingly, nearly all studies involving RASSF1A to date are related to cancer biology with few reports related to the cardiovascular field.

RASSF1A is ubiquitously expressed and has been detected in heart tissue [3, 37, 38]. Initial investigation into the role of *Rassf1* gene products in a cardiac context came from the Neyses laboratory [39]. Their findings demonstrated that both RASSF1A and RASSF1C could associate with the sarcolemmal calcium pump, PMCA4b, in neonatal rat cardiac myocytes. This interaction was shown to mediate the inhibition of ERK, and subsequent Elk transcription and suggested the possibility that RASSF1A could modulate cardiac myocyte growth [39].

3.1. $Rassf1a^{-/-}$ Mice. Five years later, the same group demonstrated that RASSF1A does in fact negatively regulate cardiac hypertrophy *in vivo* using $Rassf1a^{-/-}$ mice [37]. Although these mice have increased susceptibility to spontaneous tumorigenesis [36], no apparent cardiovascular phenotype was observed under basal conditions, that is, no differences in heart size, morphology, or function compared to WT. However, when $Rassf1a^{-/-}$ mice were challenged with pressure overload, they responded with an exaggerated hypertrophic response, evidenced by significantly greater increases in heart weight/body weight and hypertrophic gene expression (ANP, BNP, β-MHC). Cardiac myocytes of $Rassf1a^{-/-}$ mice were significantly larger, which explains the augmented heart growth. Chamber dilation of $Rassf1a^{-/-}$ mouse hearts was observed by echocardiography, consistent with eccentric hypertrophic remodeling. Hemodynamic analysis of WT and $Rassf1a^{-/-}$ mice showed a rightward shift in PV loops following pressure overload in $Rassf1a^{-/-}$ hearts, yet dP/dt_{max}, dP/dt_{min}, and fractional shortening were not altered in $Rassf1a^{-/-}$ mice compared to WT.

To examine RASSF1A function in cardiac myocytes, Oceandy et al. utilized a neonatal rat cardiac myocyte (NRCM) culture and the forced expression of RASSF1A through adenoviral gene transfer [37]. Increased RASSF1A expression inhibited phenylephrine-(PE-) induced cardiac myocyte growth and suppressed Raf-1 and ERK1/2 activation by PE

treatment. Conversely, both Raf-1 and ERK1/2 phosphorylation were increased in $Rassf1a^{-/-}$ hearts following pressure overload, suggesting negative regulation of MAPK signaling by RASSF1A. Deletion mutants of RASSF1A revealed an important function of the N-terminus of RASSF1A that disrupts the binding of active Ras and Raf-1, thus preventing ERK activation and cardiac myocyte growth.

3.2. Cardiac Myocyte-Specific Rassf1a Deletion. To better understand the function of RASSF1A in cardiac myocytes *in vivo*, we crossed genetically altered mice harboring a floxed *Rassf1a* allele [35] with mice harboring the Cre recombinase transgene driven by the α-MHC promoter. This strategy disrupted endogenous *Rassf1a* gene expression and ensured cardiac myocyte specificity [38, 40]. Similar to the $Rassf1a^{-/-}$ mice, $Rassf1a^{F/F}$*-Cre* mice had no obvious baseline cardiac phenotype. Although we also found exaggerated heart growth in the $Rassf1a^{-/-}$ mice in response to pressure overload, the $Rassf1a^{F/F}$*-Cre* mice unexpectedly had attenuated hypertrophy, that is, smaller hearts and cardiac myocytes, compared to $Rassf1a^{F/F}$ and α-MHC-Cre controls [38]. Furthermore, $Rassf1a^{F/F}$*-Cre* mice had significantly less fibrosis and myocyte apoptosis, and better cardiac function following pressure overload. This was in stark contrast to the $Rassf1a^{-/-}$ mice, which presented significantly more fibrosis and a decline in cardiac function comparable to the levels found in WT mice.

As an alternative approach we also generated two different cardiac-specific transgenic mouse lines: the first expressing wild-type RASSF1A and the second expressing a RASSF1A SARAH domain point mutant (L308P) that renders it unable to bind Mst1 [41]. Interestingly, we found that increased RASSF1A expression in the heart caused increases in Mst1 activation, cardiac myocyte apoptosis, and fibrosis, and led to worsened function following pressure overload. Conversely, RASSF1A L308P TG mice had significant reductions in Mst1 activation, apoptosis and fibrosis, while cardiac function was preserved after stress [38]. These opposing phenotypes strongly implicate Mst1 as a critical effector of RASSF1A-mediated myocardial dysfunction.

In cultured NRCMs, increased RASSF1A expression elicited activation of Mst1 and caused Mst1-mediated apoptosis. However, in primary rat cardiac fibroblasts, RASSF1A had a more pronounced effect on inhibition of cell proliferation rather than survival. Indeed, we found that silencing of RASSF1A in fibroblasts caused increased cell proliferation. Additionally, RASSF1A depletion led to an upregulation of NF-κB-dependent TNF-α expression and secretion in cardiac fibroblasts, while no change in IL-1β, IL-6, or TGF-β1 was observed. Through conditioned medium transfer experiments, we demonstrated that TNF-α secretion from fibroblasts promotes cardiac myocyte growth. Furthermore, treatment of $Rassf1a^{-/-}$ mice with a neutralizing antibody against TNF-α was able to rescue the augmented heart growth and fibrosis observed following pressure overload [38]. These data strongly implicated TNF-α as a critical paracrine factor influencing the cardiac myocyte growth response to stress *in vivo*. This work also demonstrated

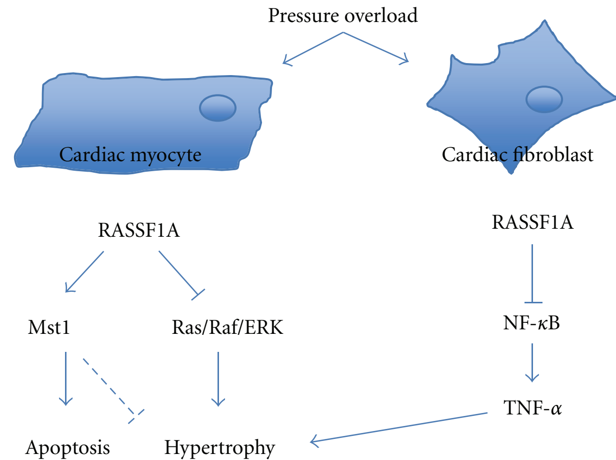

Figure 1: In cardiac myocytes, RASSF1A can prevent hypertrophy through disruption of Ras/Raf-1/ERK MAPK signaling. RASSF1A can also activate Mst1 to elicit apoptosis. In cardiac fibroblasts, RASSF1A represses NF-κB transcriptional activity and inhibits TNF-α production and secretion, thereby preventing paracrine-mediated hypertrophic signaling between fibroblast and myocyte.

the cell-type specificity of RASSF1A signaling in the heart and highlighted a novel signaling pathway downstream of RASSF1A/Mst1 that mediates a paracrine effect *in vivo* (see Figure 1). This mechanism involving multiple cell types, and paracrine signaling among them is rather unique and contrasts with more established signaling paradigms of cardiac hypertrophy including calcineurin/NFAT, HDAC/MEF2 and MEK/ERK pathways, which have been elucidated in the cardiac myocyte [42].

3.3. Hippo Signaling in the Heart. Our previous work has demonstrated the functional importance of Hippo signaling in the heart. Using genetically altered mouse models we showed that increased expression of Mst1, and subsequent activation of the Hippo pathway, caused increased apoptosis, dilated cardiomyopathy, and premature death [43]. Interestingly, expression of Mst1 also attenuated cardiac myocyte hypertrophy thereby impairing the heart's ability to appropriately respond to stress. In contrast, expression of a kinase-inactive Mst1 mutant (DN-Mst1) prevented cell death and protected the heart from insult [43]. Lats1/2 kinases (mammalian homologs of Warts) are targets of Mst1/2 that can phosphorylate and inactivate Yap, thereby inhibiting Yap-mediated gene transcription [44]. Similar to our findings related to Mst1, we demonstrated that transgenic expression of Lats2 in the heart led to inhibited growth and worsened function [45]. Conversely, kinase-inactive Lats2 (DN-Lats2) transgenic mice had larger hearts both at baseline and following pressure overload and displayed attenuated cardiac myocyte apoptosis in response to stress [45]. Taken together, these results provide further evidence that activation of Hippo signaling, via increased Mst1 or Lats2 expression, inhibits cardiac myocyte growth and promotes apoptosis in the adult heart. Furthermore, selective inhibition of Hippo signaling in the cardiac myocyte (DN-Mst1 or DN-Lats2

TG) confers protection against insult, similar to what we observed in the cardiac myocyte-specific RASSF1A deleted mice [38]. However, the hypertrophic response in these two models was opposite, which may result from a Hippo-independent pathway(s) downstream of RASSF1A. It should be pointed out that studies of adult mouse models using cardiac myocyte-restricted deletion of Mst1/2, Lats1/2 or Yap have not been published. Findings from these models should be helpful in further elucidating the role of Hippo signaling components in the adult murine heart.

Recent work from the Martin laboratory demonstrated the importance of mammalian Hippo signaling during cardiac development and cardiac myocyte proliferation [46]. Conditional deletion of Salvador (Sav1) in the embryonic heart, driven by Nkx2.5-Cre expression, caused increased myocyte proliferation and cardiac enlargement and was mediated by hyperactivation of Yap and subsequent Wnt/β-catenin-regulated gene expression. In a similar vein, direct targeting of Yap expression in the developing mouse heart further demonstrated its role in governing both myocyte proliferation and heart growth [47]. Interestingly, both reports described an interaction between Yap and Wnt signaling, highlighting additional Hippo signaling crosstalk in the heart.

4. Conclusion

Fueled by the initial reports described herein, investigation into the role of RASSF1A in cardiovascular biology has begun to accelerate. Yet many questions remain outstanding. Among them, what are the upstream inputs that regulate RASSF1A function? What is the mechanism responsible for RASSF1A cell-type-specific signaling? What are the molecular constituents of the RASSF1A complex? Does RASSF1A have additional Mst1-independent functions in the heart, as has been demonstrated in tumor cell lines [41]? Recent work identified activated K-Ras as a promoter of RASSF1A signaling in colorectal cancer cells [48]. This finding begs the question of whether K-Ras or additional Ras isoforms regulate RASSF1A in other systems and cell types. Based on our findings in *Rassf1a*-deleted mice [38], we speculate that the difference in proliferative capacity between cardiac myocytes and fibroblasts may explain the distinct effects of RASSF1A signaling in the heart. There may also be differences in the expression or localization of signaling components, thereby modulating their ability to effectively signal in certain cell types. Exposure to diverse signals and cues in the extracellular milieu may also contribute to varied outcomes downstream RASSF1A.

As we continue to elucidate the role of RASSF1A and Hippo signaling in the heart, its importance in cardiac development, physiology, and disease is becoming apparent. Of course, translating these findings into meaningful therapeutic strategies remains the greatest challenge. Our work has shed light on the importance of cell type specificity RASSF1A in determining pathological outcomes [38]. We also defined a paracrine mechanism functioning downstream of RASSF1A in response to cardiac stress [38]. It is likely that additional complexities remain to be uncovered and will ultimately influence possible interventions to manipulate RASSF1A and treat heart disease.

RASSF1A signaling is diverse and our knowledge regarding RASSF1A function is rapidly expanding. Given that a bridge from cancer to cardiovascular biology is in place, it is likely that as additional RASSF1A mechanisms of action are discovered, its impact on cardiac biology will continue to grow.

Acknowledgments

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Research Article

The SARAH Domain of RASSF1A and Its Tumor Suppressor Function

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The Ras association domain family 1A (RASSF1A) tumor suppressor encodes a Sav-RASSF-Hpo domain (SARAH), which is an interaction domain characterized by hWW45 (dSAV) and MST1/2 (dHpo). In our study, the interaction between RASSF1A and RASSF1C with MST1 and MST2 was demonstrated and it was shown that this interaction depends on the SARAH domain. SARAH domain-deleted RASSF1A had a similar growth-reducing effect as full-length RASSF1A and inhibited anchorage independent growth of the lung cancer cell lines A549 significantly. In cancer cells expressing the SARAH deleted form of RASSF1A, reduced mitotic rates ($P = 0.001$) with abnormal metaphases ($P < 0.001$) were observed and a significantly increased rate of apoptosis was found ($P = 0.006$) compared to full-length RASSF1A. Although the association with microtubules and their stabilization was unaffected, mitotic spindle formation was altered by deletion of the SARAH domain of RASSF1A. In summary, our results suggest that the SARAH domain plays an important role in regulating the function of RASSF1A.

1. Introduction

The Ras association domain family 1 gene (*RASSF1)* was identified on chromosome 3p21.3, a region frequently deleted in cancer [1]. There are two major transcripts of *RASSF1*, termed *RASSF1A* and *RASSF1C*, which are transcribed from different CpG island promoters [1]. The promoter of *RASSF1A* is often hypermethylated in cancer, whereas the promoter region of *RASSF1C* is never methylated [2, 3]. Both isoforms encode a Ras association domain in the C-terminus, an ATM-kinase phosphorylation site, a SARAH protein interaction domain, and the N-terminal sequence of *RASSF1A* harbors a diacyl glycerol binding domain [1, 4]. It has been demonstrated that *RASSF1A* encodes a tumor suppressor gene, which reduces tumor growth *in vivo* and *in vitro* [1, 5–8]. Deletion of *Rassf1a* in mice significantly increased spontaneous and induced tumorigenesis [9–11]. It has been reported that RASSF1A binds to microtubules and protects cells from microtubule destabilizing agents [7, 12–15]. This interaction contributes to cell cycle regulation and mitotic progression.

RASSF1A is regulated by the binding of RAS and the novel Ras effector 1 (NORE1) and mediates proapoptotic signals through binding of the mammalian sterile 20-like kinase 1 and 2 (MST1 and MST2) [16–19]. Moreover, an association of RASSF1A with the BH3-like protein modulator of apoptosis was observed and this interaction regulates conformational change of BAX and apoptosis [20, 21]. RASSF1A promotes MDM2 self-ubiquitination and prevents p53 degradation [22]. Additionally, it was reported that RASSF1A inhibits the anaphase promoting complex (APC) through its binding to CDC20 and induces mitotic arrest by stabilizing

mitotic cyclins [23] and it was further shown that the Aurora mitotic kinases are involved [24]. However, we were not able to verify the interaction between RASSF1A and CDC20 [25].

In the C-terminal part of RASSF1A and RASSF1C, a protein-protein interaction domain called SARAH (Sav/RASSF/Hpo) has been determined [26]. The SARAH domain is a key feature of the Hippo signaling pathway components, by which the interaction of Sav, Rassf, and Hpo is accomplished [26]. In the Drosophila Hippo pathway, Salvador (Sav, the human homologue is named WW45) acts as a scaffold protein that interacts with the proapoptotic kinase Hippo (Hpo, human homologue MST) [27–29]. Hpo is able to phosphorylate the kinase Warts (human homologue LATS), which in *Drosophila* leads to cell cycle arrest and apoptosis [28–30].

It was shown that the single *Drosophila* orthologue of the human RASSF proteins restricts Hpo activity by competing with Sav for binding to Hpo [31]. Praskova et al. previously showed that human RASSF1A interacts with MST1 through the C-terminus [16] and more precisely through the SARAH domain [32]. MST1 has two caspase 3 cleavage sites and both MST1 and MST2 play a role in processes of apoptosis both before and after caspase cleavage [33]. The cleaved form of MST1 translocates in the nucleus and phosphorylates histone H2B at Ser14 [34, 35]. H2B phosphorylation correlates with apoptotic chromatin condensation and nuclear fragmentation in mammalians and yeast [35, 36]. Following death receptor activation, MST1 (homologue of Hpo) is known to become activated through caspase-dependent cleavage [19]. The cleaved fragment then localizes from the cytoplasm to the nucleus, where it induces apoptosis [19] by chromatin condensation through activation of the c-Jun N-terminal kinase pathway [37].

Both RASSF1A and WW45 activate MST2 by promoting its autophosphorylation [38]. Moreover, RASSF1A stabilizes MST1/2 activation by preventing the dephosphorylation of these kinases [39]. Activated MST1/2 phosphorylates different targets including LATS kinases, which in turn activate the transcription coactivator YAP1 [40, 41]. Other MST1/2 targets are H2AX [42], FOXO [43], and troponin [44].

To gain new insights into the tumor suppressor function of RASSF1A, we deleted its SARAH domain and analyzed its altered function. Deletion of the SARAH domain resulted in a decreased colony formation of tumor cells. During mitosis, abnormal spindle formation was observed. We demonstrate that the interaction of RASSF1A and RASSF1C with MST1 and MST2 depends on the SARAH domain. Deregulation of the SARAH domain may contribute to altered proapoptotic and mitotic signaling of RASSF1A.

2. Materials and Methods

2.1. Tissues and Cell Lines. The localization experiments and the protein expression experiments were performed in HEK293 and COS7 cells (ATCC, Manassas, Virginia, USA). For stable transfection, the lung cancer cell line A549 (ATCC) was used. A549 cells harbor epigenetic silenced RASSF1A, but express RASSF1C [1].

2.2. Interaction Studies Using the Yeast Two-Hybrid System. The Matchmaker Two-hybrid system (Clontech, Mountain View, USA) was utilized. cDNAs of RASSF1A and RASSF1C were described previously [1]. The genes MST1 and MST2 were cloned after amplification of the fragments from EST-clones IRAKp961C0282Q and IRAKp961I0613Q (RZPD, Berlin, Germany), respectively. RASSF1AΔSARAH and RASSF1A were cloned into the vector pGADT7, RASSF1C and RASSF1CΔSARAH into the vector pAS2-1, and MST1 and MST2 into pGBKT7 [25, 45]. Mutant forms of RASSF1 were generated with the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) and forward primer (5′-AGGAAAATGACTCTGGGCCCCTTGGGTGACCTCT) and the complementary reverse primer. All constructs were confirmed by sequencing. The yeast strain PJ69-4A was cotransformed with 0.1 μg of each plasmid using the PEG/LiAc method. The interaction analysis was carried out on SD minimal medium plates without adenine and histidine and the transformation efficiency was controlled on SD plates with adenine and histidine. The strength of interaction was investigated by quantification of the expression of the β-galactosidase reporter gene with o-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate at 420 nm.

2.3. Interaction Studies by Coprecipitation. MST1 and MST2 were cloned into the vector pCMV-Tag1 (Stratagene, La Jolla, USA) and/or in the vector pEBG. To investigate the interaction of specific RASSF1 forms, MST1 and MST2, cotransfections (Lipofectamine 2000, Invitrogen, Carlsbad, USA) were performed in HEK293 cells. Plasmids (pEBG and pCMV-Tag 1) were used, that express GST-Flag-RASSF1A, GST-Flag-RASSF1C, GST-Flag-RASSF1AΔSARAH, GST-Flag-RASSF1CΔSARAH, and GST or Flag-MST1, Flag-MST2, and Flag-WW45 [45]. Two days after transfection, total protein was extracted in RIPA buffer. The GST-fused proteins were precipitated with glutathione-sepharose (Amersham Biosciences, Freiburg, Germany). Samples were separated on a 10% PAGE gel and blotted. The interaction was determined with anti-Flag-antibodies (F3165, Sigma, Steinheim, Germany) and anti-GST antibodies (Santa Cruz, Santa Cruz, USA).

2.4. MST1 and MST2 Phosphorylation. A549 were treated with 3 μM staurosporine for 3 h or transfected with 10 μg of constructs with Turbofect for 36 h (Fermentas, St. Leon-Rot, Germany). Total protein was isolated using Flag-lysis buffer, samples were denatured with Laemmli-buffer, separated in 10% SDS-PAGE, and blotted onto PVDF membranes. First antibodies are: anti-GAPDH (FL332 Santa Cruz, USA), anti-P-MST1 (Thr183)/MST2 (Thr180) (#3681 Cell signaling, Frankfurt, Germany), anti-Flag (F3165 Sigma, Steinheim, Germany), and secondary antibodies are HRP coupled (sc2004/5 Santa Cruz, USA). ECL (WBKLS0100 Millipore, Schwalbach, Germany) was used for detection with Versadoc (Bio-Rad, München, Germany).

2.5. Generation of Stable Transfected Cell Lines. RASSF1A, RASSF1AΔSARAH, and RASSF1C were cloned into the vector pCMV-Tag1 (Stratagene, La Jolla, USA). The lung cancer cell line A549 was transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, USA). Colonies were selected under 1 mg/mL Geneticin (Gibco, Karlsruhe, Germany) in DMEM and clones were picked after 4 weeks. Expression of Flag-RASSF1 was confirmed by RT-PCR using the FLAG-specific primer (5′-TGGATTACAAGGATGACGACG) and RASSF1-specific primer L27111 (5′-TCCTGCAAGGAGGGTGGCTTC). PCR products were analyzed on a 2% Tris-borate EDTA agarose gel.

2.6. Proliferation Analyses of Stable Transfected Cell Lines. Growth curves of stable transfected clones were analyzed by seeding 150,000 cells in triplicates in 6-well plates. Every 24 hours, cells were counted using a Neubauer counting chamber. In order to investigate the proliferation in soft agar, stable transfected cells were seeded in 0.3% agarose. Experiments were performed in duplicates with 5,000 cells per plate under selection with 1 mg/mL Geneticin. Colony size was measured after 4 weeks with a microscope (LEICA DMIRB, Wetzlar, Germany). Therefore, colonies were stained with 400 μL of 5 mg/mL INT and the size of 25 colonies was determined with MetaVue (Molecular Devices GmbH, Ismaning, München).

2.7. Localization Studies. RASSF1A and RASSF1C were cloned into the fluorescence vector pEYFP-C2 (Clontech, Mountain View, USA). The deletion of the SARAH domain of RASSF1A and RASSF1C was accomplished by the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) with the upper SARAH deletion primer 5′-AGGAAAATGACTCTGGGCCCCTTGGGTGACCTCT and the complementary lower primer. After transient transfection into HEK293 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, USA), the localization of YFP-RASSF1A, YFP-RASSF1AΔS, YFP-RASSF1C, and the vector control were investigated with a fluorescence microscope. Cells were costained with anti-α-tubulin (Molecular Probes, Invitrogen, Carlsbad, USA) antibodies and Alexa Fluor goat anti-mouse (Molecular Probes, Invitrogen, Carlsbad, USA) antibodies to show the colocalization with the microtubules and spindle poles, respectively. Nuclei of the cells were visualized by staining with DAPI (0,1 μg/mL in PBS). Cells in mitoses were scored by microscopy (ZEISS Axioplan 2). Cells with highly condensed chromosomes and spindle structures were classified as mitotic cells. For microtubule stability analysis, the cells were treated one day after transfection for one hour with 20 μM nocodazole, fixed, and stained with DAPI and anti-α-tubulin antibodies. YFP constructs are shown in green color.

2.8. Apoptosis Analysis by TUNEL Staining. The lung cancer cell line A549 was transiently transfected with different RASSF1A constructs tagged with yellow fluorescence (pEYFP-C2) using Lipofectamine 2000 (Invitrogen, Carlsbad, USA). After two days, the transfected cells were harvested and centrifuged on a slide. After fixation with formaldehyde, a TUNEL staining was performed with the *In Situ* Cell Death Detection Kit, TMR red (Roche, Mannheim, Germany). The nuclei were costained with DAPI (0,1 μg/mL) solution. Slides were quantified with a fluorescence microscope (ZEISS Axioplan 2). Yellow fluorescence expressing cells (500) were counted and the rate of apoptotic cells (red fluorescence) was calculated. All experiments were done in triplicates.

2.9. Statistical Analysis. All statistical evaluations were performed using the SPSS 12.0 Software (SPSS Science, Chicago, IL). A Probability of $P < 0.05$ was considered as significant.

3. Results

3.1. The RASSF1-SARAH Domain Binds MST1 and MST2. In silico analysis of RASSF1A (340 aa) and RASSF1C (270 aa) by PROSITE (www.expasy.org) revealed the presence of a Sav-RASSF-Hpo (SARAH) domain at their C-terminus (290 to 337 and 220 to 267, resp.) (Figure 1(a)). To gain insight into the function of the SARAH domain of RASSF1, the interaction of RASSF1A, RASSF1C, RASSF1AΔSARAH, and RASSF1CΔSARAH with MST1 and MST2 was investigated in the yeast two-hybrid system (Figures 1(b) and 1(c)). Both RASSF1 isoforms (RASSF1A and RASSF1C) interacted with MST1 and MST2, but when the SARAH domain was deleted the proteins were not able to interact anymore. These interactions were quantified in an o-nitrophenyl-β-D-galactopyranoside (ONPG) assay (Figure 1(c)). The interaction of RASSF1A and RASSF1C with MST1 and MST2 was verified by coprecipitation experiments (Figure 1(d) and data not shown). The interaction between RASSF1A and MST1 and MST2 was confirmed (Figure 1(d)). There was also an interaction between RASSF1C and MST1 and MST2 (data not shown). Interaction of RASSF1A and RASSF1C with MST1 and MST2 was abolished, when the SARAH domain was deleted (Figure 1(d) and data not shown).

3.2. RASSF1A Constructs with Deletion of the SARAH Domain Inhibit Cell Growth. Growth effects of the SARAH domain of RASSF1 were investigated in stable transfected lung cancer cells. The lung cancer cell line A549 was transfected with RASSF1A, RASSF1C, and RASSF1AΔS, and the control vector (pCMV-Tag1) and the growth of these cells were evaluated (Figure 2). A549 harbor epigenetically silenced RASSF1A but express RASSF1C. We picked stable transfected colonies and expression of *RASSF1*-specific forms was confirmed by RT-PCR (Figure 2(a)). Subsequently, the proliferation- and anchorage-independent growth of these clones was analyzed. Proliferation of RASSF1A expressing cells was significantly reduced at 96 h compared to RASSF1C expressing cells and control cells ($P = 0.022$ and $P = 0.007$, resp.). Two RASSF1AΔS expressing clones showed a similar growth to RASSF1A expressing cells. RASSF1AΔS clone1 and clone2 had a significant reduction of growth at 96 h compared to RASSF1C ($P = 0.027$ and 0.042, resp.) and controls ($P = 0.018$ and 0.029, resp.). Subsequently, the anchorage independent growth was determined in soft agar experiments

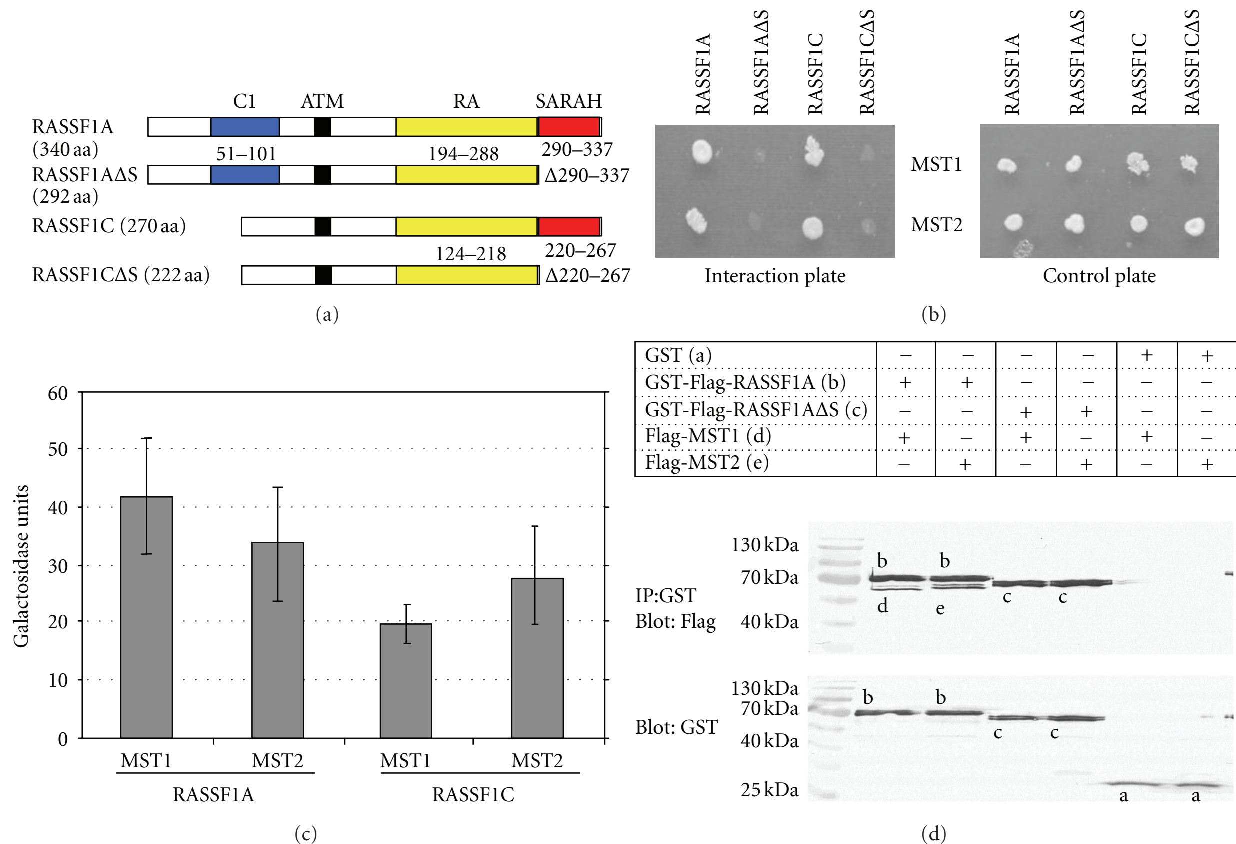


Figure 1: Binding studies of RASSF1, MST1, and MST2. (a) Characteristic domains of RASSF1 isoforms and SARAH deletion (ΔS) mutants are the protein kinase C conserved region (C1; blue), the ATM-kinase phosphorylation site (black), Ras-association (RalGDS/AF-6) domain (RA; yellow), and the Sav-RASSF-Hpo interaction site (SARAH; red). (b) Interaction analysis using the yeast two hybrid system. The indicated constructs were cotransformed into yeast strain PJ 69-4A. Interaction was evaluated on SD plates without alanine and histidine (interaction plate). Transformation was controlled on SD plates with alanine and histidine (control plate). (c) Quantitative interaction analysis using the ONPG assay. In three independent colonies, the activation of the β-galactosidase reporter gene was quantified with ONPG as substrate. The standard deviation is indicated. (d) Binding studies in coprecipitation. Constructs that express GST (a), GST-Flag-RASSF1A (b), GST-Flag-RASSF1AΔS (c), Flag-MST1 (d), or Flag-MST2 (e) were transfected into HEK293 cells. After two days, total protein was extracted and GST-tagged proteins were precipitated with glutathione sepharose. Samples were separated on a 10% PAGE gel and blotted. The precipitated and coprecipitated proteins were determined with anti-Flag-antibodies and anti-GST antibodies.

(Figures 2(c) and 2(d)). In these experiments, cells expressing RASSF1AΔS (clone1) exhibited a significantly reduced colony growth (average colony size: 22 μm) compared to RASSF1A (average: 36 μm; $P < 0.01$, Welch's test) and control (average: 66 μm; $P < 0.01$, Welch's test).

3.3. Expression of RASSF1A with a Deleted SARAH Domain Induces Aberrant Mitosis and Apoptosis. We transfected yellow fluorescent protein-tagged RASSF1A and RASSF1AΔSARAH (RASSF1AΔS) into HEK293 and COS7 cells and the localization was determined by fluorescence microscopy (Figure 3(a)). RASSF1A and RASSF1AΔS are both localized at the tubulin-containing cytoskeleton during interphase. In mitotic cells, RASSF1A and RASSF1AΔS were detected at spindles and centrosomes (Figure 3(a)). In the RASSF1AΔS expressing HEK293 cells, multipolar spindles and unequal alignment of the chromosomes between poles were observed (Figure 3(a)). In COS7 cells, overexpression of RASSF1AΔS also induced monopolar spindles (Figure 3(a)). Interestingly, mitotic rate of RASSF1AΔS expressing cells was significantly ($P = 0.001$) reduced to 3.4% compared to 8.6% in RASSF1A transfected HEK293 cells (Figure 3(b)). The mitotic rate in vector transfected cells was 1.2% (data not shown). The majority (74.3%; $P < 0.001$) of mitoses in RASSF1AΔS expressing cells were abnormal (multi- or monopolar) compared to RASSF1A transfected cells, where only 0.6% of abnormal mitosis were counted (Figure 3(c)).

To determine if the aberrant spindle formation in RASSF1AΔS expressing cells is due to an altered microtubule stability of these cells, transiently transfected cells were treated with 20 μM nocodazole for one hour (Figure 4(a)). In YFP control cells, this treatment caused massive depolymerisation of the microtubules in interphase and during mitosis. RASSF1A, RASSF1AΔS, and RASSF1C overexpressing cells were able to stabilize microtubules from depolymerization by nocodazole (Figure 4(a) and data not shown).

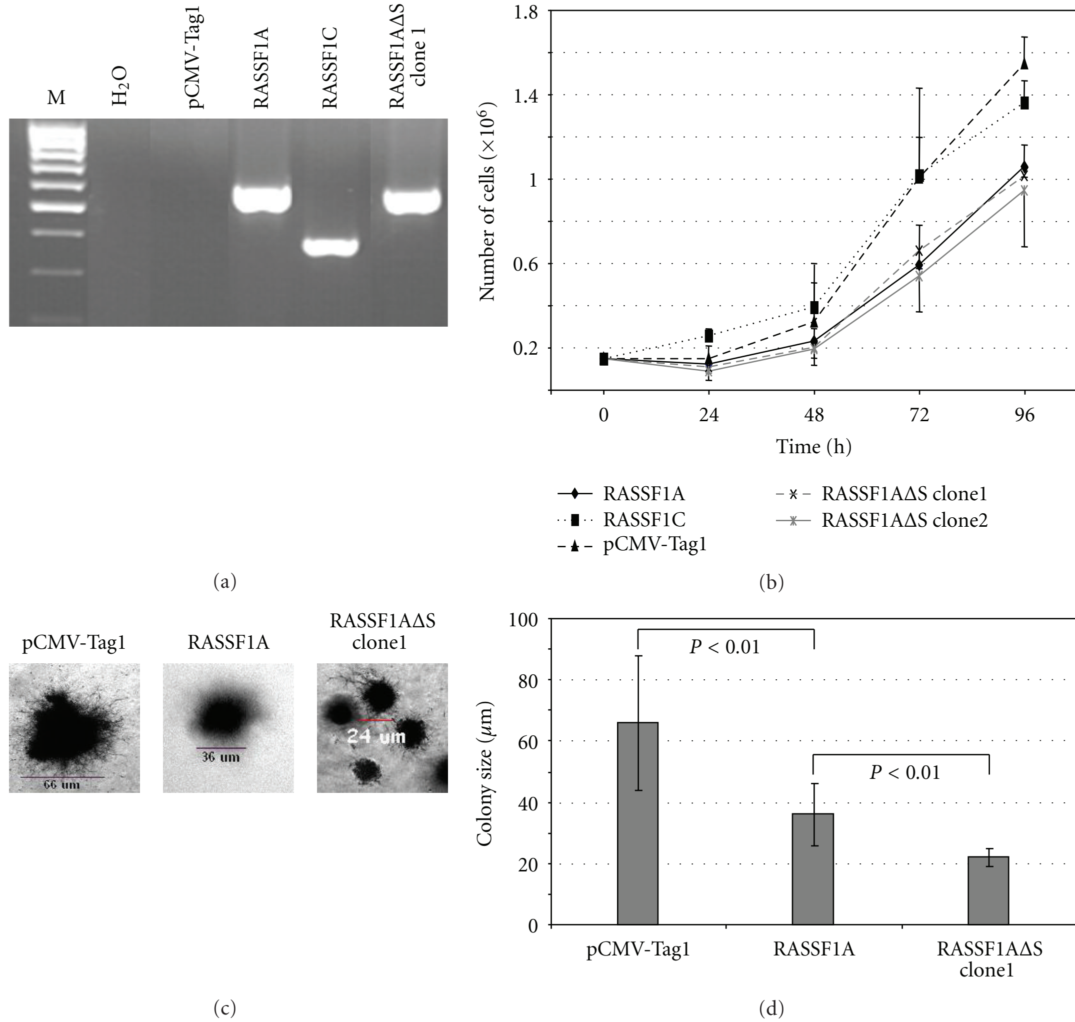
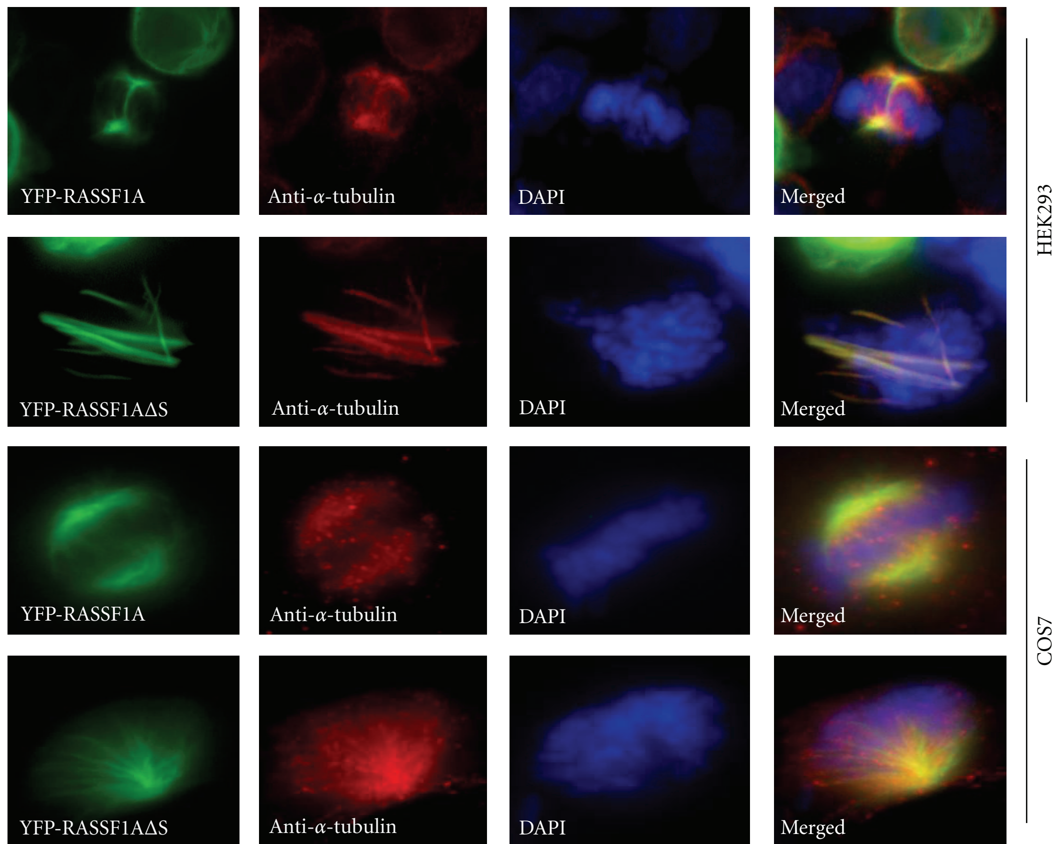


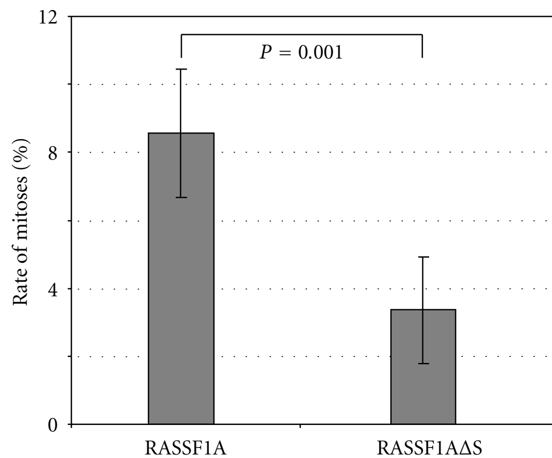
Figure 2: Proliferation analysis of RASSF1AΔSARAH expressing lung cancer cells. (a) A549 lung cancer cells were transfected with pCMV-Tag1, RASSF1A, RASSF1C, and RASSF1AΔSARAH (RASSF1AΔS) and stable clones were analyzed. RASSF1 expression of clones was confirmed by RT-PCR using a Flag-specific forward primer and primer L27111. Products (RASSF1A and RASSF1AΔS, 585 bp; RASSF1C, 374 bp) were analysed with controls (pCMV-Tag1 and H_2O) and a 100 bp ladder (M) on a 2% Tris-borate EDTA agarose gel. (b) Growth curve of A549 cells stably transfected with the indicated constructs. Clones were analyzed by seeding 1.5×10^5 cells in 6-well plates. Every 24 hours, cells were counted using a Neubauer chamber. Three independent experiments were performed and the mean and standard deviation is plotted. (c) Colony sizes in a soft agar experiment after 4 weeks. Examples of colonies expressing pCMV-Tag1, RASSF1A, and RASSF1AΔS are shown. (d) 25 colonies were measured and the average colony size was calculated. Statistical significance P-values are indicated.

To analyze the effect of the deletion of the SARAH domain on the proapoptotic function of RASSF1A, a transient transfection into the lung cancer cell line A549 was performed and the rate of transfected and apoptotic cells was calculated after 1 to 2 days (Figure 4(b)). Apoptotic cells were stained using red fluorescence TUNEL-Kit and YFP was used as a control. The rate of apoptosis was significantly ($P = 0.001$) higher, when the cells expressed RASSF1A (28%) in comparison to RASSF1C (16%) and the YFP control (14%, Figure 4(b)). The deletion of the SARAH domain (RASSF1AΔS) resulted in a significantly increased apoptotic rate of 39% compared to RASSF1C ($P < 0.001$) and RASSF1A ($P = 0.006$, Figure 4(b)). In summary, our results show that expression of RASSF1A with a deletion of the SARAH domain deregulates normal mitotic progression and enhances apoptosis.

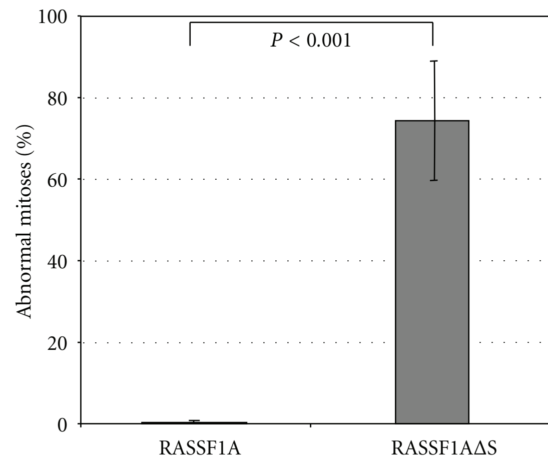
Subsequently, RASSF1A-induced autophosphorylation of MST1 and MST2 was analyzed in A549 lung cancer cells (Figure 5). For this propose, we have utilized an antibody that detects endogenous MST1 and MST2 only when phosphorylated at Thr183 and Thr180, respectively. Treatment of A549 cells with 3 μM staurosporine induced phosphorylation of MST1 and MST2 (Figure 5), as described previously [34, 46]. However, when A549 cells were transfected with RASSF1A and RASSF1AΔSARAH, phosphorylation of MST1 and MST2 was not detected (Figure 5). Similar results were



(a)



(b)



(c)

Figure 3: Effects of RASSF1AΔSARAH expression on mitosis. (a) HEK293 and COS7 cells were transfected with YFP-RASSF1A and YFP-RASSF1AΔSARAH (RASSF1AΔS) and stained using DAPI and anti-α-tubulin antibody. Yellow fluorescent is shown in green. (b) Rate of mitosis in HEK293 after transient expression. Cells with highly condensed chromosomes and spindle structures were classified as mitotic cells (c) Abnormal mitoses (monopolar, multipolar spindles and abnormal spindle fibers) were counted in HEK293 cells and the rate of abnormal mitosis is plotted. All experiments were done in triplicates and 500 cells each were evaluated. The mean and standard deviation were determined. Statistical significant P-values are indicated.

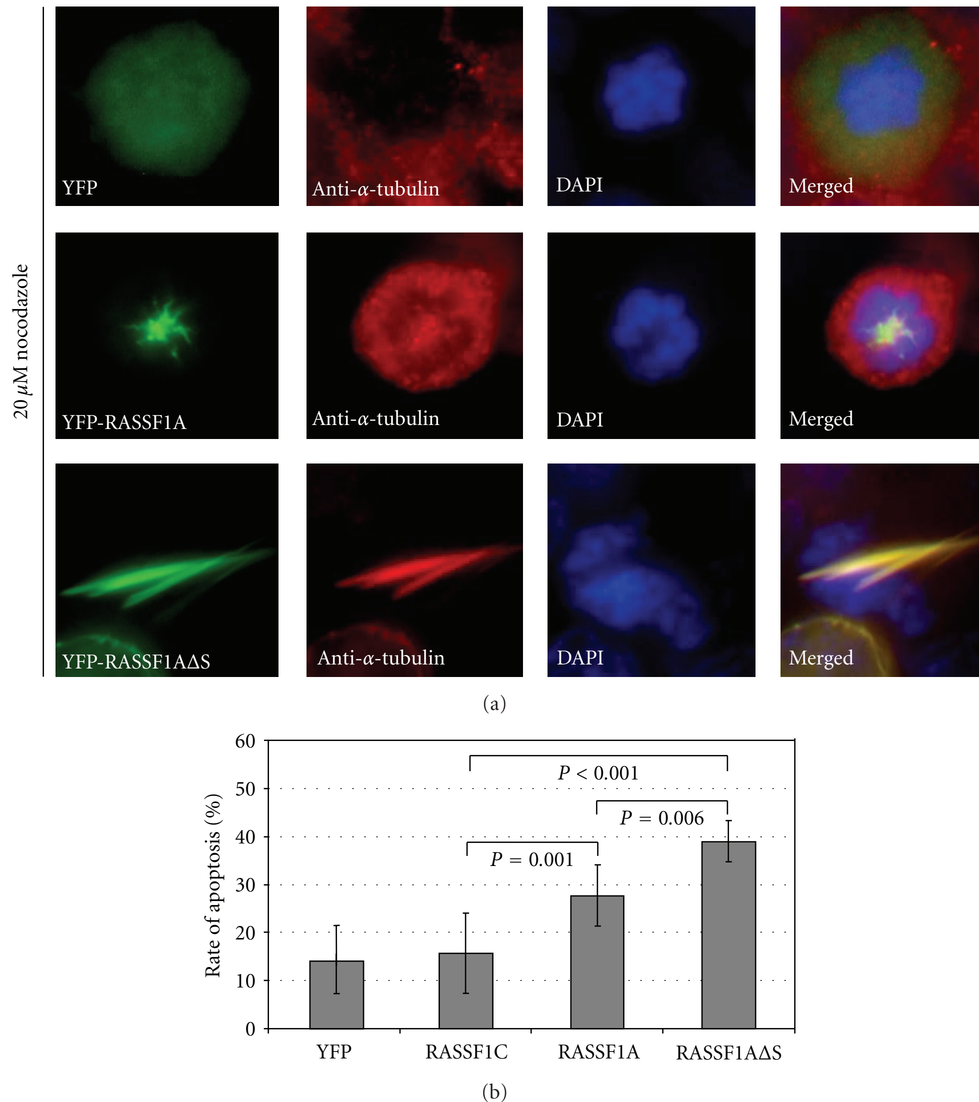


Figure 4: (a) Microtubule stability of RASSF1AΔSARAH expressing mitotic cells. HEK293 cells were transfected with plasmids YFP, YFP-RASSF1A or YFP-RASSF1AΔSARAH (YFP-RASSF1AΔS). One day after transfection, the cells were treated for one hour with 20 μM nocodazole, fixed and stained with DAPI and anti-α-tubulin antibodies. Yellow fluorescence is shown in green. (b) Induction of apoptosis after transient expression of RASSF1A and RASSF1AΔSARAH. Lung cancer cells A549 were transfected with YFP, YFP-RASSF1C (RASSF1C), YFP-RASSF1A (RASSF1A), and YFP-RASSF1AΔSARAH (RASSF1AΔS). TUNEL staining was utilized to determine the frequency of apoptotic cells in transfected cells. All experiments were done in triplicates and the mean and standard deviation were determined. Statistical significant P-values are indicated.

obtained in HEK293 cells (data not shown). Praskova et al. showed that the MST1 kinase autoactivation through phosphorylation is inhibited by coexpression of RASSF1A and RASSF1C [16].

4. Discussion

RASSF1A is a tumor suppressor gene, which is involved in several signaling pathway including apoptosis, microtubule stability, proliferation, and mitotic regulation [2, 3]. In our study, we have analyzed the function of the Sav-RASSF1-Hpo (SARAH) domain of RASSF1A. Here, we report that the SARAH domain regulates several pathways, which are frequently altered in tumors. The SARAH domain is involved in apoptosis and growth-suppressing functions of RASSF1A like anchorage-independent proliferation. Moreover, the SARAH domain is important for mitotic progression and spindle formation. It has been previously reported that RASSF1 interacts through its C-terminal domain with MST1 and thereby regulates MST1-mediated apoptosis [16, 18, 19]. We demonstrate that RASSF1A and RASSF1C interact with both MST1 and MST2. This complex may regulate several pivotal signaling pathway including apoptosis and phosphorylation of Warts/LATS serine threonine kinases that regulate mitotic progression.

It has been reported that RASSF1A regulates a proapoptotic pathway through its interaction with the proto-oncogene Ras and the novel Ras effector 1 (Nore1) [18, 32].

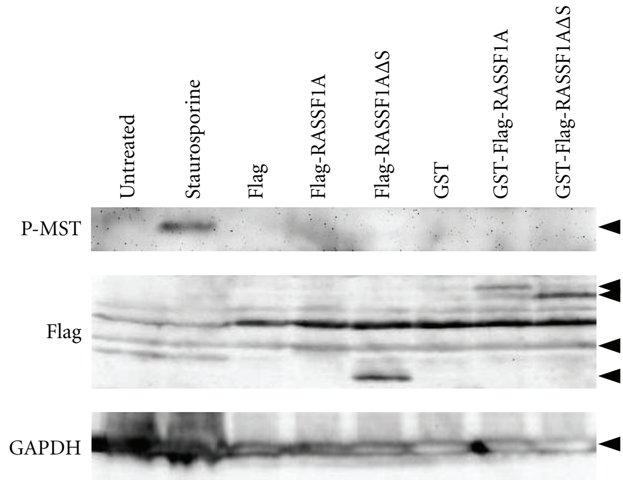


Figure 5: Phosphorylation of MST1/2 is induced by staurosporine. A549 were treated with 3 μM Staurosporine for 3 h. For transfections Turbofect (Fermentas) was used with 10 μg of indicated constructs for 36 h. Total protein was isolated using Flag-lysis buffer, samples were denatured with Laemmli-buffer, separated in 10% SDS-PAGE and blotted onto PVDF membrane. First antibodies are: α-GAPDH, α-P-MST1 and α-Flag and secondary antibody are HRP coupled (sc2004 and sc2005 Santa cruz). ECL (WBKLS0100 Millipore) was used for detection with Versadoc (Biorad). Arrowheads indicate top down: P-MST, GST-Flag-RASSF1A, GST-Flag-RASSF1AΔSARAH (ΔS), Flag-RASSF1A, Flag-RASSF1AΔSARAH (ΔS), and GAPDH.

RASSF1A and Nore1 interact with the proapoptotic Ste20 protein kinase MST1 via the C-terminus [16, 19] through the SARAH domain [32]. The homologue of RASSF1, NORE1 forms a complex with MST1 that mediates a proapoptotic pathway induced by Ras [18]. Early on, it was reported that MST1 is a serine/threonine protein kinase that could autophosphorylate itself [47] and later Praskova et al. demonstrated that MST1 phosphorylates and activates itself, whereas this autophosphorylation is inhibited when MST1 is bound to RASSF1A and RASSF1C [16]. We show that the interaction of RASSF1A and RASSF1C with MST1 and MST2 depends on the C-terminal SARAH domain. Since the SARAH domain binds the proapoptotic kinases MST1 and MST2, a deregulation of these kinases may contribute to the apoptotic rate in the cells with truncated RASSF1A. However, we could not observe an autophosphorylation of MST1/2 after transfection of RASSF1A and RASSF1AΔSARAH. In contrast, staurosporine induced strong phosphorylation of MST1/2. This indicates that RASSF1A-induced apoptosis observed in A549 cells occurs MST independent, possibly through the N-terminus of RASSF1A, that associates with MDM2 and death-domain-associated protein (DAXX) and contributes to p53 activation in response to DNA damage [22]. Alternatively, RASSF1A was further linked to apoptosis through interacting with the microtubule-associated protein C19ORF5 [48, 49]. Furthermore, Donninger et al. described RASSF1A to interact with the potential tumor suppressor Salvador to promote apoptosis independently of Hippo signaling by modulating p73 [50].

It was demonstrated that RASSF1A colocalizes with the microtubule network during interphase and is found at the spindles and centrosomes during mitosis [14, 23]. RASSF1A binds to tubulin [7], thereby stabilizing microtubules [7, 12–14] and regulating the mitotic progression. RASSF1A overexpression leads to a mitotic arrest at metaphase [14], to a G1 arrest [51], to a G2/M arrest [52], to a G1 and G2/M arrest [7], and a prometaphase arrest [23]. The domain required for both microtubule association and stabilization was mapped to an amino-acid fragment from 120 to 288 [14]. Thus, the microtubule binding site and the SARAH domain are different [12, 14, 53] and this is consistent with our observation. Rong et al. showed that the microtubule binding was lost upon mutation of the phosphorylation site 203 in RASSF1A [54]. However, we and others did not observe an altered microtubule binding using phosphomimicking or nonphosphorylatable mutants of RASSF1A [15, 24].

RASSF1A was also reported to interact with MAP1B (microtubule-associated protein 1B) and C19ORF5 (chromosome 19 open reading frames 5), both microtubule-associated proteins [13, 49, 53]. C19ORF5 is a hyperstabilized microtubule-specific binding protein of which accumulation causes mitochondrial aggregation and cell death [48]. Regarding C19ORF5, it was demonstrated that its knockdown led to mitotic abnormalities [55], that C19ORF5 localizes to centrosomes, and it was stated that C19ORF5 is required for the recruitment of RASSF1A to the spindle poles [53, 55]. Liu et al. reported that RASSF1A caused hyperstabilization of microtubules and the accumulation of C19ORF5 on them [48]. The complex LATS1/MST2/WW45 is found together with RASSF1A at the centrosome, and it was shown that defects in this pathway may lead to abnormal mitosis caused by cytokinesis failure. Thus, RASSF1A may mediate organization of mitotic spindle poles through the recruitment of MST and LATS to the centrosomes.

In summary, our data indicate that RASSF1A is important for several signals, which are frequently altered in tumorigenesis, including apoptosis, mitotic spindle organization, and proliferation. Our data suggest that other domains (e.g., microtubule association domain) than SARAH also significantly contribute to the proapoptotic and antiproliferative function of RASSF1A. Specific interaction of RASSF1A with MST/LATS and other binding partners (e.g., RAS, MDM2, DAXX, C19ORF5, and Salvador) might be important in the regulation of proliferation and apoptosis and in the formation of normal mitotic spindles and processes of dividing chromosomes by RASSF1A.

Abbreviations

RASSF1A:	Ras association domain family 1A
MST:	Mammalian STE20 like kinase
LATS:	Large tumor suppressor
WW45:	45 kDa WW domain protein
Sav:	Salvador
Hpo:	Hippo
SARAH domain:	Sav-RASSF-Hpo interaction domain
RT-PCR:	Reverse transcriptase PCR
YFP:	Yellow fluorescence protein
TUNEL:	Terminal transferase mediated dUTP nick end labeling.

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Research Article

RASSF1A and the Taxol Response in Ovarian Cancer

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The RASSF1A tumor suppressor gene is frequently inactivated by promoter methylation in human tumors. The RASSF1A protein forms an endogenous complex with tubulin and promotes the stabilization of microtubules. Loss of RASSF1A expression sensitizes cells to microtubule destabilizing stimuli. We have observed a strong correlation between the loss of RASSF1A expression and the development of Taxol resistance in primary ovarian cancer samples. Thus, we sought to determine if RASSF1A levels could dictate the response to Taxol and whether an epigenetic therapy approach might be able to reverse the Taxol resistant phenotype of RASSF1A negative ovarian tumor cells. We found that knocking down RASSF1A expression in an ovarian cancer cell line inhibited Taxol-mediated apoptosis and promoted cell survival during Taxol treatment. Moreover, using a combination of small molecule inhibitors of DNA Methyl Transferase enzymes, we were able restore RASSF1A expression and Taxol sensitivity. This identifies a role for RASSF1A in modulating the tumor response to Taxol and provides proof of principal for the use of epigenetic therapy to overcome Taxol resistance.

1. Introduction

RASSF1A is a poorly understood tumor suppressor that can modulate the cell cycle, tubulin dynamics and apoptosis [1–3]. It is subjected to epigenetic inactivation at high frequency in a broad range of human tumors, including approximately 50% of ovarian tumors [1, 4, 5]. Overexpression of RASSF1A promotes hyperstabilization of microtubules reminiscent of Taxol [6, 7], and previous investigations have shown that loss of RASSF1A sensitizes cells to microtubule destabilizing drugs such as nocodazole [7]. Thus, RASSF1A appears to play an important role in modulating microtubule stabilization. This implies that the RASSF1A levels in a tumor cell may impact how the cell responds to Taxol treatment. The development of resistance to Taxol remains a serious problem in the treatment of ovarian cancer.

The most frequent mechanism by which RASSF1A is inactivated in tumors is by hypermethylation promoter leading to transcriptional silencing [1, 4, 5]. Thus, the gene remains intact, just dormant. Over recent years, a series of small molecules have been identified that can inhibit the DNA methylation system and restore expression of genes that have suffered aberrant promoter methylation [8]. This has given rise to the concept of epigenetic therapy, whereby a tumor would be treated with drugs to restore the expression and function of RASSF1A or some other epigenetically inactivated target. If RASSF1A plays a key role in the response to Taxol, epigenetic therapy could be potentially serve as an approach to overcome the resistance.

In an attempt to address the issue of RASSF1A expression and Taxol resistance, we measured the expression levels of RASSF1A in a series of primary ovarian tumor samples that were characterized for resistance or sensitivity to Taxol. The results showed a very strong correlation between the reduced relative expression of RASSF1A and Taxol resistance in primary ovarian cancer. We then used an shRNA-based approach to generate a matched pair of ovarian tumor cell lines that were positive or negative for RASSF1A expression. In this system, loss of RASSF1A impaired the ability of Taxol to promote microtubule polymerization and rendered the cells resistant to the growth inhibitory effects of Taxol. Using an epigenetic therapy approach, we found that reactivating RASSF1A expression in a RASSF1A-negative ovarian tumor cell line enhanced the sensitivity of the cells to Taxol. Thus

we confirm the hypothesis that RASSF1A plays a role in the cellular response to Taxol and provide proof of principal for the use of epigenetic therapy as strategy to address the problem of Taxol resistance ovarian cancer.

2. Materials and Methods

2.1. Tissue Culture. A547 and UCI-107 cells were grown in DMEM/10% FBS. Cells were transfected with shRNA constructs described previously [9] using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) using the manufacturers protocol and selected in 1 μg/mL puromycin. Cells were treated with Taxol (Sigma, St. Louis, MI, USA) at the described doses for 48 hours prior to assay. Cell numbers were measured by trypsinization and counting in a haemocytometer. Cells were treated with Zebularine [10] and/or RG108 [11] dissolved in DMSO for 48 hours prior to assay. t-tests were used to determine statistical significance.

2.2. Quantitative Real-Time PCR. qRT-PCR analysis was used to evaluate the expression of RASSF1A in primary ovarian tumors essentially as described previously [12] using the following primers to RASSF1A: forward, 5′-GGACGAG-CCTGTGGAGTG-3′, and reverse, 5′-TGATGAAGCCTGT-GTAAGAACC-3′. β-actin was used as the reference gene. Sequences of the β-actin primers have been previously described [13].

2.3. Western Blotting. Cells were lysed in modified RIPA buffer as described previously [14], and subjected to Western analysis using an RASSF1A polyclonal antibody described previously [6]. Tubulin antibodies were purchased from Santa Cruz biochemical (Santa Cruz, CA, USA). Protein concentrations in lysates were measured prior to loading using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Densitometry was performed using a Densitometer and Quantity One software. Values are expressed as adjusted volume Optical Density units/mm^2.

2.4. Caspase Assays. Cells were plated in 12-well plates at 30% confluency and treated with Taxol the next day. 22 hours later cells were lysed and assays with the Caspase-Glo kit (Promega, Madison, WI, USA) as described by the manufacturer.

3. Results

3.1. RASSF1A Downregulation Correlates with Acquisition of Taxol Resistance in Primary Ovarian Tumors. mRNA isolated from the tumors of patients with stage III or IV papillary serous ovarian cancer [12] whose tumors were either responsive or nonresponsive to Taxol were assayed by qRT-PCR for the levels of RASSF1A expression. Ten samples were used for each group and the data expressed as fold change relative to RASSF1A expression in the nonresponder group, after normalization to the expression of β-actin. Those tumors which responded to Taxol showed considerably higher levels of RASSF1A mRNA than those which were resistant (Figure 1).

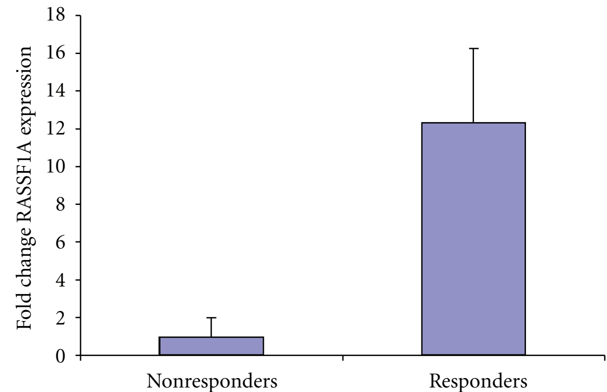

Figure 1: RASSF1A downregulation correlates with acquisition of Taxol resistance in primary ovarian tumors: qRT-PCR analysis of primary ovarian tumors correlates loss of RASSF1A expression with the development of Taxol resistance. Left column is relative expression of RASSF1A in Taxol-resistant patients; right column is relative expression in Taxol-sensitive patients. Data is expressed as fold change relative to the nonresponder group after normalization to β-actin expression. t-test was used to determine P was <.05.

3.2. RASSF1A Knockdown Induces Resistance to Taxol. UCI-107 cells are a Taxol-sensitive ovarian cancer cell line [15]. We transfected the cells with our validated RASSF1A shRNA [9] or the empty vector and generated a stable matched pair by selection in puromycin. The cells were then western blotted for RASSF1A using our polyclonal rabbit antibody [6]. Figure 2(a) shows that RASSF1A expression was effectively knocked down in the shRNA transfected cell line.

The matched pair system was then challenged with Taxol for 48 hours and cell survival measured. Loss of RASSF1A enhanced the survival of the treated cells (Figure 2(b)). RASSF1A is a proapoptotic protein and loss of RASSF1A expression may induce resistance to apoptosis [9]. To determine if that may be the case in ovarian cancer cells treated with Taxol, we then examined the effects of RASSF1A expression on apoptosis after Taxol treatment. The RASSF1A ± UCI-107 cells were treated with Taxol for 22 hours and then assayed for apoptosis using the Promega Caspase 3/7 kit, which is a fluorescent measure of caspase activation. Figure 2(c) shows that downregulation of RASSF1A promotes resistance to apoptosis induced by Taxol. We also observed a very slight reduction in the basal levels of caspase activation in the cells transfected with the RASSF1A shRNA.

3.3. Loss of RASSF1A Reduces the Ability of Taxol to Promote Microtubule Polymerization. RASSF1A binds microtubules and promotes their stabilization/polymerization [6, 7, 16]. Indeed, the effects of overexpressing RASSF1A in cells on tubulin is reminiscent of the effects of treating them with Taxol [6]. Moreover, downregulation of RASSF1A makes cells more sensitive to Nocodazole, a microtubule destabilizing drug [7]. Thus, we hypothesized that the presence of RASSF1A may be important to the ability of Taxol to induce microtubule polymerization. This would confirm RASSF1A loss as a component of the development of Taxol resistance in ovarian cancer and explain the results obtained in Figure 1.

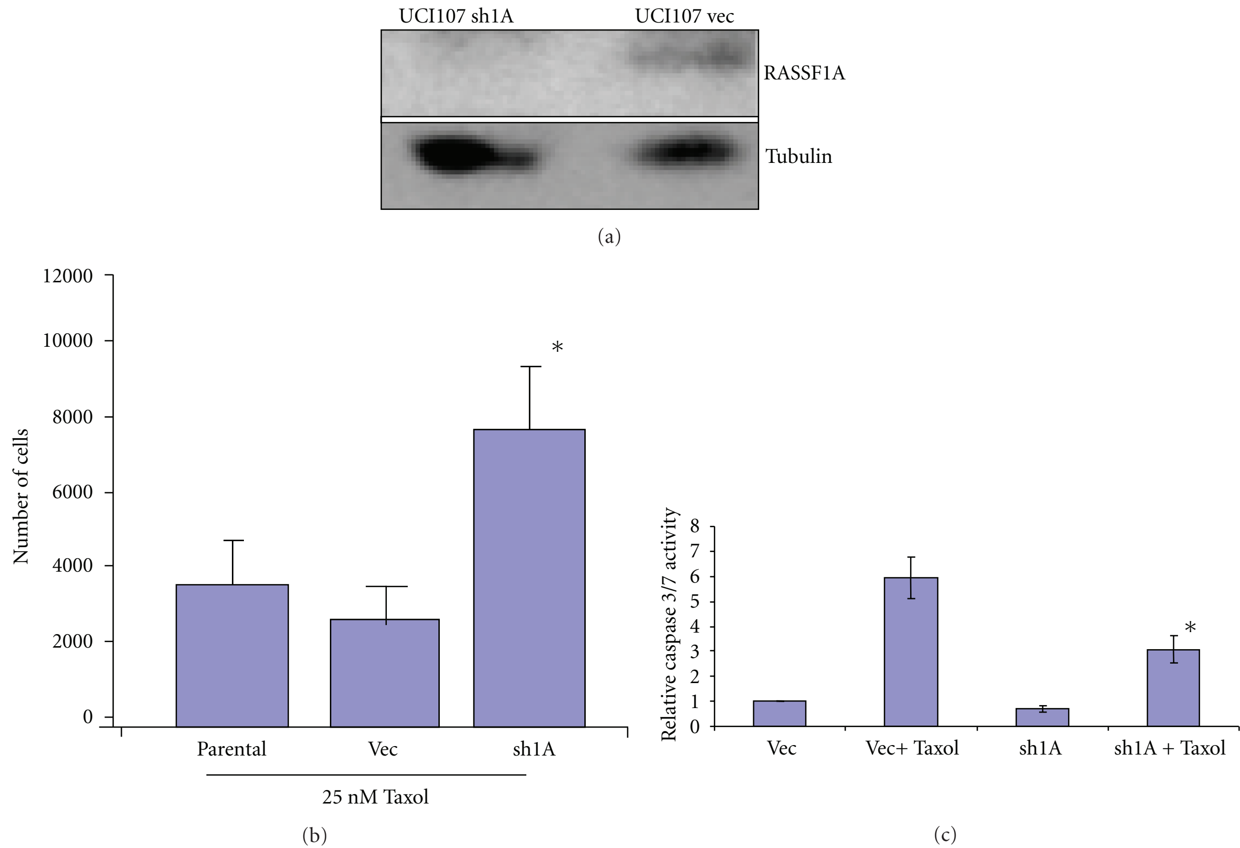

Figure 2: Loss of RASSF1A confers resistance to taxol-mediated apoptosis. A matched pair of RASSF1A ± cells was generated by stably knocking down RASSF1A expression in UCI-107 ovarian cancer cells using a RASSF1A-specific shRNA. Knockdown of RASSF1A was confirmed by western blotting. Tubulin served as a loading control (a). The UCI-107 RASSF1A ± cells were grown to 50% confluency and then treated with 25 nM Taxol or vehicle control 48 hours and cell number determined (b). Data represent an average of triplicate experiments, $^*P < 0.1$ compared to parental or vector control cells. (c). The RASSF1A ± UCI-107 cells were treated with 25 nM Taxol for 22 hours and caspase activation measured as a readout for apoptosis using a luminescent caspase activation assay. Data represent the average of two assays performed in triplicate. *, statistically different from vector control cells treated with taxol, $P < 0.05$.

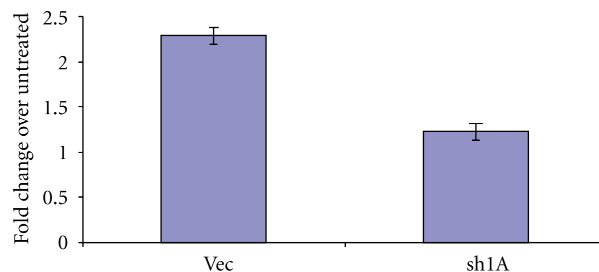

Figure 3: The ability of Taxol to promote tubulin acetylation is dependent on RASSF1A. The UCI-107 RASSF1A ± matched pair was treated with Taxol for 48 hours, cell lysates prepared and equal amounts of protein subjected to western blotting using antibodies specific for total or acetylated tubulin. The relevant bands from the western blot were quantified and average data from three experiments expressed as a ratio of acetylated tubulin to total tubulin to give a fold change. Knockdown of RASSF1A resulted in an approximately 50% reduction in the relative acetylation of tubulin, $P = 0.042275$.

When Taxol polymerizes, it becomes acetylated and this has been used as a marker for polymerization [17]. The UCI-107 RASSF1A ± matched pair of cell lines was treated with Taxol. After 48 hours the cells were lysed and equal quantities of protein subjected to Western analysis first for total tubulin and then for acetylated tubulin using an acetylated tubulin specific antibody. The ratio of acetylated tubulin to total tubulin was determined by densitometric scanning of the western blots to permit quantitative assessment of the effects of the presence of RASSF1A. Figure 3 shows that loss of

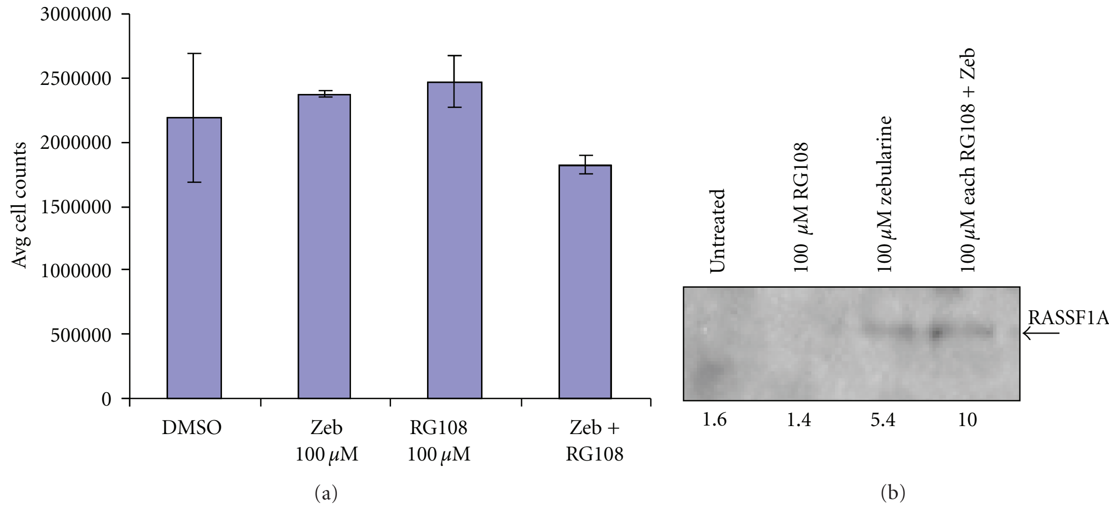

Figure 4: Synergistic reactivation of RASSF1A expression by RG108 and Zebularine. (a). RASSF1A negative A547 ovarian cancer cells were treated with DMSO, Zebularine, RG108 or Zebularine and RG108 in combination for 48 hours and surviving cells counted as a measure of toxicity. Treatment with either of the demethylating agents resulted in no significant difference in cell number. (b). A547 cells were treated with the indicated doses of RG108 and Zebularine alone or in combination for 48 hours and cell lysates prepared. Equal amounts of proteins were immunoprecipitated with an anti-RASSF1A antibody and the immunoprecipitates subjected to Western analysis for RASSF1A. Densitometric quantification of the bands is shown below the figure.

RASSF1A expression reduces the ability of Taxol to promote microtubule polymerization.

3.4. Synergistic Restoration of RASSF1A Expression with DNMT Inhibitors. To examine the possibility that small molecule-induced restoration of RASSF1A expression might affect the cellular response to Taxol, we used the ovarian cancer cell line A547 that is negative for RASSF1A expression and exposed it to treatment with the DNA Methyl Transferase (DNMT) inhibitors Zebularine [10] and RG108 [11]. Zebularine has previously been shown to be active in restoring RASSF1A expression but is more specific and hence less toxic than the first generation DNMT inhibitor 5-AzaC [11, 18]. RG108 is a novel DNMT inhibitor that was designed to specifically inhibit the enzyme DNMT1 [19]. We also used the two in combination. Examination of the toxicity of RG108 and Zebularine allowed the determination of the minimal dose that provoked no detectable changes in cell growth or morphology. Combination of these two doses also resulted in no overt cell death (Figure 4(a)). Western analysis showed that Zebularine was more effective than RG108 at restoring RASSF1A expression but in combination their effects were greater than additive (Figure 4(b)).

3.5. Combined Epigenetic Therapy Restores Taxol Sensitivity. Having determined that RG108 and Zebularine could act synergistically to restore RASSF1A expression at doses that were too low to induce cell toxicity, we examined the effect of the treatment on the Taxol response of the cells. Figure 5 shows that A547 cells pretreated with the Zebularine/RG108 epigenetic therapy regimen exhibited an enhanced sensitivity to Taxol.

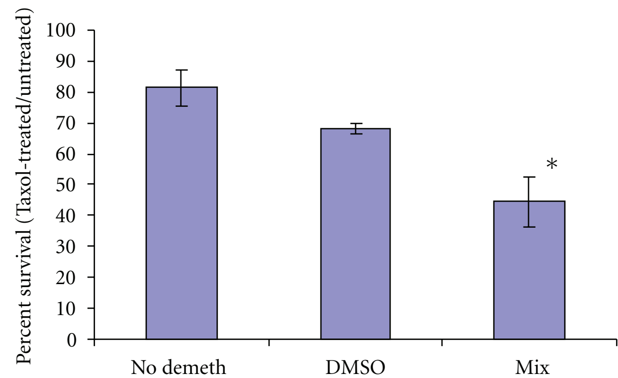

Figure 5: Synergistic epigenetic therapy enhances the taxol response of ovarian tumor cells. A547 cells were treated with carrier (DMSO) or a combination of RG108 and Zebularine (mix) for 48 hours, after which 400 nM Taxol was added and the cells incubated for an additional 48 hours. The number of viable cells was determined by trypan blue staining. Data are expressed as percent surviving cells relative to non-Taxol-treated cells for each condition.

4. Discussion

The RASSF1A tumor suppressor is frequently inactivated by an epigenetic process of aberrant promoter methylation in ovarian cancer [1]. RASSF1A complexes with microtubules and enhances their polymerization. Inactivation of RASSF1A results in an increased sensitivity to microtubule destabilizing drugs. Overall, the data suggests that RASSF1A plays an important role in the stabilization of microtubules. As the drug Taxol is thought to work in large part by stabilizing microtubules, we hypothesized that loss of RASSF1A expression might play a role in the development of resistance

to Taxol. Our analysis of primary ovarian tumors showed that RASSF1A levels were much lower on average in Taxol resistant tumors. Based on this supporting evidence we proceeded to generate a matched pair of ovarian tumor cell lines that were identical other than for RASSF1A expression. Using this system, we showed that loss of RASSF1A expression caused a significant increase in the resistance of the cells to growth inhibition and apoptosis induction by Taxol.

These data supported the idea that if we could restore RASSF1A expression then we might be able to restore Taxol sensitivity to a tumor cell. Using a combination of demethylating drugs we were able to restore RASSF1A expression. These drugs, RG108 and Zebularine, appear much less toxic than the established demethylating drug 5-Aza-C, even when used in combination (unpublished observation, G. Clark). The cells with restored RASSF1A expression proved much more sensitive to Taxol. Thus, we provide proof of principle for the use of epigenetic therapy to overcome Taxol resistance in ovarian cancer. Moreover, the methylation of the RASSF1A promoter might serve as a predictive marker for the effectiveness of Taxol based therapy.

These studies focused on the role of RASSF1A in the Taxol response because of the apparent role of RASSF1A in supporting microtubule polymerization. However, RASSF1A has a general role in apoptosis and has now been shown to play a role in DNA repair. Thus, RASSF1A restoration might also be expected to enhance the effects of drugs which act by inducing apoptosis and DNA damage. Indeed, Zebularine has been shown to enhance the effects of Cisplatin in ovarian cancer models [20].

In these studies, we used Zebularine and RG108 as DNMT inhibitors. As they have different mechanisms of action, we hypothesized that they might have a synergistic activity. This would appear to be the case. As better agents arise that are more specific, for example Nanaomycin [21], the effectiveness and practicality of this strategy is likely to increase.

RASSF1A exhibits an SNP, which is present in excess of 20% of the Caucasian population. This SNP produces a variant protein where Alanine 133 is substituted for a serine. The A(133)S variant protein is defective for interacting with certain isoforms of tubulin [22] and is defective for binding the microtubule association protein MAP1a [23]. Mutations close to this SNP can impair the ability of RASSF1C to promote microtubule polymerization [6]. Thus, it may be interesting to determine if the presence of this SNP may also affect the response of an individual to Taxol treatment.

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Review Article

Pathogenetic and Prognostic Significance of Inactivation of RASSF Proteins in Human Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is one of the most frequent solid tumors worldwide, with limited treatment options and a dismal prognosis. Thus, there is a strong need to expand the basic and translational research on this deadly disease in order to improve the prognosis of HCC patients. Although the etiologic factors responsible for HCC development have been identified, the molecular pathogenesis of liver cancer remains poorly understood. Recent evidence has shown the frequent downregulation of Ras association domain family (RASSF) proteins both in the early and late stages of hepatocarcinogenesis. Here, we summarize the data available on the pathogenetic role of inactivation of RASSF proteins in liver cancer, the molecular mechanisms responsible for suppression of RASSF proteins in HCC, and the possible clinical implications arising from these discoveries. Altogether, the data indicate that inactivation of the RASSF1A tumor suppressor is ubiquitous in human liver cancer, while downregulation of RASSF2 and RASSF5 proteins is limited to specific HCC subsets. Also, the present findings speak in favour of therapeutic strategies aimed at reexpressing *RASSF1A*, *RASSF2*, and *RASSF5* genes and/or inactivating the RASSF cellular inhibitors for the treatment of human liver cancer.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent tumors, with 0.25-1 million of newly diagnosed cases each year worldwide [1–3]. HCC burden is not distributed evenly throughout the world. Indeed, more than 80% of HCC cases occur in sub-Saharan Africa and Eastern Asia, whereas a much lower HCC incidence characterizes North and South America, Northern Europe, and Oceania [1–3]. Nonetheless, HCC frequency is rapidly growing in low-rate areas as well. In the latter geographic areas, such a rise in HCC occurrence is the result of a combination of factors, including an increasing incidence of cirrhosis caused by alcohol intake, hepatitis C virus (HCV) and hepatitis B (HBV) chronic infection, as well as a general improvement in survival among cirrhosis patients, who are then at risk of developing HCC [1–3]. Furthermore, the rapidly growing number of cryptogenic cirrhosis and HCC cases might be due to a severe form of nonalcoholic fatty liver disease, namely, the nonalcoholic steatohepatitis (NASH) [2].

HCC is a rapidly lethal disease, with an average life expectancy of about 6 months from the time of the diagnosis [1–3]. Like most other solid tumors, surgery plays a fundamental role in its treatment. Surgical resection, local ablation therapies, and liver transplantation are regarded as potentially curative treatment modalities for HCC. However, these therapeutic approaches can be applied only to a limited number of HCC patients since the diagnosis is most often done in the late stages of the disease [1–3]. Furthermore, therapies with pharmacological agents or alternative strategies do not substantially improve the prognosis of patients in which HCC is unresectable [1–3]. Targeted therapies are an innovative and emerging tool to selectively kill cancer cells while sparing the normal, unaffected tissue and thus might be useful for the treatment of human HCC. The effectiveness of targeted therapies against HCC has been recently envisaged by the significantly higher survival length of patients treated with the multikinase inhibitor Sorafenib compared with that of patients treated with placebo [4]. However, the increased life expectancy of Sorafenib-treated

patients is limited to about three months, implying that Sorafenib alone cannot substantially modify the prognosis of patients with advanced HCC [4]. This emphasizes the need to investigate the contribution of different signaling pathways to tumor development and progression in human HCC in order to identify novel prognostic markers and molecular targets for its early diagnosis, chemoprevention, and treatment.

Although epidemiologic studies have identified the major risk factors, the molecular pathogenesis of HCC remains largely unknown. It is presumed that development and progression of HCC are the consequence of cumulative genetic and epigenetic events, similar to those described in other solid tumors [5]. Among the most frequently involved tumor suppressor genes in HCCs are *pRb*, *p53*, *M6P/IGF2* receptor, and *E-cadherin* [2, 5]. Oncogenic activation of *c-Myc*, *Cyclin D1*, and *β-catenin* genes has also been detected in various subsets of HCC [2, 5]. Importantly, unrestrained activation of the Ras/mitogen-activated protein kinase (MAPK) pathway seems to play a major role both in liver malignant transformation and tumor progression [6]. Ras proteins are members of a family of small-guanosine-triphosphate-(GTP-) regulated molecular switches for signaling pathways that modulate cell growth, survival, and migration [7, 8]. Once activated, Ras induces the protein kinase activity of RAF kinase. Raf phosphorylates and activates MAPK kinase kinase (MEK), which subsequently phosphorylates and activates extracellular signal-regulated kinase (ERK), ultimately leading to the upregulation of downstream targets involved in cell proliferation, survival, migration, and invasion [7, 8]. Recent studies have shown that Ras/MAPK pathway is upregulated by multiple factors in HCC in the absence of Ras mutations, including downregulation of the Ras GTPase activating proteins, loss of the ERK inhibitor dual specificity phosphatase 1 (DUSP1), and inactivation of members of the Ras association domain family (RASSF) proteins [6, 9, 10]. Thus, it is plausible to believe that downregulation of cellular inhibitors of Ras may be a key and alternative mechanism leading to the propagation of the Ras signaling in a context of wild-type *Ras* in human HCC.

2. RASSF Proteins Status in Human HCC

Due to the widely recognized tumor suppressive role of RASSF1A in carcinogenesis [11–15], numerous studies have investigated its levels in human liver cancer and related them to the clinicopathological parameters. RASSF1A was found to be frequently and progressively downregulated in nontumorous surrounding livers, dysplastic nodules, and HCC when compared with normal (disease-free) livers, with the lowest levels being detected in the tumors. In most investigations, reduced levels of RASSF1A were found to be ubiquitous in HCC regardless of the etiologic factors associated with tumor development (hepatitis B or C chronic infection, alcohol consumption, exposure to food contaminated by aflatoxin B1, etc.), strongly suggesting that universal inactivation of RASSF1A in liver cancer is required for hepatocarcinogenesis [6, 16–22]. Of note, it has been shown [23] that inactivation of *RASSF1A* gene by promoter hypermethylation is already a frequent event in liver fibrosis and cirrhosis, conditions that often precede the development of HCC, but not in hepatocellular adenoma (HCA). Thus, the latter study suggests that RASSF1A hypermethylation occurs early during hepatocarcinogenesis and could be useful as a marker to help discriminating between HCA and HCC [23]. Furthermore, a recent report indicates that reduced RASSF1A protein expression is related to TNM stage, alpha-fetoprotein levels, and the presence of metastasis, portal vein emboli, capsular infiltration, and multiple tumor nodes, implying that assessment of RASSF1A levels might be helpful also for prognosis prediction in human HCC [24].

Different from RASSF1A, the other main RASSF1 isoform (RASSF1C) was found to be expressed at similar levels in normal livers, HCC, and corresponding nontumorous surrounding livers [6]. As concerns RASSF2, its downregulation was detected only at tumor stage and was closely associated with elevated serum alpha-fetoprotein level, but not significantly with clinical stage and hepatic fibrosis [25, 26]. Levels of the main isoforms of RASSF5 (also known as novel Ras effector 1 or NORE1) were also investigated in HCC. The analysis of a large panel of HCC samples showed that NORE1A levels were significantly lower in liver tumors characterized by a poorer outcome (as defined by a patients' length of survival shorter than 3 years after partial liver resection) when compared with HCC with a better prognosis (survival longer than 3 years) [6]. Thus, downregulation of NORE1A seems to be involved in liver tumor progression and biologic aggressiveness. Low levels of the other RASSF5 isoform, NORE1B, were also described in about 60% of the investigated HCC [27]. In the latter tumor collection, downregulation of the *NORE1B* gene did not correlate with tumor grade or stage or the etiology of the disease [27]. Based on this body evidence, it is tempting to speculate that NORE1A and NORE1B may possess distinct biologic activities in liver (cancer) cells.

3. Regulation of Expression and Activity of RASSF Proteins

RASSF1A levels and activity are presumably regulated by numerous and complex mechanisms in HCC. These mechanisms include epigenetic silencing of the gene and posttranslational modifications of the protein, which affect RASSF1A stability and half-life. In accordance with a pioneering study on lung cancer and several cancer-derived cell lines, including the HepG2 hepatoma cells [28], epigenetic *RASSF1A* suppression by hypermethylation of its promoter has been suggested as the main mechanism contributing to RASSF1A downregulation in several cancer types, including HCC [6, 11–24]. In the latter disease, promoter hypermethylation of *RASSF1A* gene was shown to occur both in nonneoplastic surrounding livers and HCC, with higher degree of methylation being detected at tumor stage [6, 16–24]. In particular, an intriguing study showed that, in the hepatitic liver (affected by chronic hepatitis and/or cirrhosis, dysplastic nodules, or HCC), the *RASSF1A* gene

promoter was extensively methylated, with a methylation degree that increased from regenerative conditions (cirrhosis) to dysplastic nodules, to HCC [21]. Of note, the level of methylation at *RASSF1A* promoter gradually increased by ageing in the nondiseased liver as well [21]. These data suggest the existence of an age-related phenomenon leading to the development and expansion of an epigenetically methylated hepatocyte subpopulation, which might be connected to hepatocarcinogenesis. In another investigation, the *RASSF1A* gene exhibited a weak but clearly detectable methylation signal in normal liver tissue and focal nodular hyperplasia specimens in 57% and 70% of cases, respectively, using sensitive qualitative assay conditions [20]. By using a stringent threshold, none of the normal tissue or focal nodular hyperplasia specimens was methylation-positive, whereas 85% of the hepatocellular carcinoma biopsies were still positive for *RASSF1A* gene hypermethylation [20]. Furthermore, *RASSF1A* methylation degree possessed the highest discriminatory power between HCC and nonmalignant livers [20]. Although the number of investigated specimens was limited, this investigation strongly suggests the use of quantitative real-time PCR-based assays for the assessment of *RASSF1A* promoter methylation status, which might be highly helpful for the discrimination between frankly malignant and nonmalignant liver lesions. In a large-scale study conducted in China, it was found that *RASSF1A* promoter hypermethylation precedes the occurrence of other epigenetic and genetic alterations, such as hypermethylation of *p16INK4A* promoter and mutations of the *p53* gene [29]. Based on these data, the authors suggest that RASSF1A could represent a potential target in preventing malignant transformation of hepatocytes [29]. In addition, promoter methylation of *RASSF1A* gene has been frequently detected in livers affected by hereditary haemochromatosis, a predisposing condition for the development of HCC [30]. Altogether, these data substantiate the role of RASSF1A inactivation both in early and late steps of liver carcinogenesis. Of note, promoter hypermethylation at the *RASSF1A* gene CpG island was found to be frequently associated with additional epigenetic and genetic alterations. In mammary epithelial cells, it has been shown that *RASSF1A* gene inactivation is associated with deacetylation and lysine 9 trimethylation of histone H3 and an impaired binding of Sp1 at the *RASSF1A* promoter. These epigenetic events precede the occurrence of DNA methylation spreading in the *RASSF1A* promoter [31]. Thus, these data suggest that histone modifications may trigger de novo DNA methylation of the *RASSF1A* promoter in epithelial cells. Similar to that described in mammary epithelial cells, epigenetic silencing of *RASSF1A* gene was demonstrated to depend on promoter hypermethylation and histone H3-K9 methylation in human HCC samples [32]. Furthermore, loss of heterozygosity at the lung cancer tumor suppressor locus 3p21.3, where *RASSF1A* is located, was frequently detected in HCC specimens in which the *RASSF1A* promoter was hypermethylated [6, 33]. The two-hit mode (promoter hypermethylation and loss of heterozygosity) of gene inactivation has been described also for other liver tumor suppressor genes, such as *SOCS1-3* and *DUSP1* [6, 9]. As concerns the relationship between *RASSF1A* promoter hypermethylation and the clinicopathological parameters of HCC patients, it has been shown that *RASSF1A* epigenetic silencing is significantly associated with the levels of DNA adducts generated by aflatoxin B1 (AFB1), a mycotoxin with hepatocarcinogenic potential produced by the fungus Aspergillus Flavus [16]. It is important to underline the fact that AFB1 protumorigenic potential in the liver has been attributed to AFB1 mutagenic properties over the p53 tumor suppressor gene so far [34, 35]. The significant association between AFB1 adducts and *RASSF1A* epigenetic inactivation indicates that AFB1 might initiate hepatocarcinogenesis via additional molecular mechanisms independent of p53, including the suppression of the *RASSF1A* gene.

Besides epigenetic silencing and/or genetic loss, post-translational mechanisms such as microRNA-driven suppression and ubiquitin-dependent proteolysis are also involved in RASSF1A inactivation in HCC. In particular, microRNA-602 has been demonstrated to negatively regulate RASSF1A levels in the HepG2 liver cancer cell line [36]. Also, levels of microRNA-602 were inversely correlated with those of RASSF1A in normal livers, HCC, and corresponding nontumorous surrounding livers, further supporting a role of microRNA-602 in the downregulation of RASSF1A in human liver cancer [36]. Another way of RASSF1A inactivation has been originally described in various cell lines. In these cells, the S-phase kinase-associated protein 2 (SKP2), an oncogenic subunit of the Skp1-Cul1-F-box ubiquitin ligase complex, interacts with ubiquitinates and promotes the degradation of RASSF1A at the G1-S transition of the cell cycle [37]. The SKP2-dependent destruction of RASSF1A requires phosphorylation of RASSF1A on serine-203 by the cyclin D-cyclin-dependent kinase 4 [37]. In human HCC, it has been found that SKP2-dependent proteosomal degradation occurs mainly in tumors characterized by biological and clinical aggressiveness [38]. Also, SKP2-driven ubiquitination and *RASSF1A* epigenetic silencing represent two mutually exclusive mechanisms responsible for RASSF1A inactivation in human HCC [38]. The data obtained in the human HCC sample collection were further substantiated *in vitro*. Indeed, transfection of SKI human HCC cells (expressing low SKP2 levels) with wild-type *SKP2* cDNA increased the proliferation rate proportionally to SKP2 expression, concomitantly triggering downregulation of multiple tumor suppressor proteins, including $P21^{WAF1}$, $P27^{KIP1}$, $P57^{KIP2}$, P130, FOXO1, and RASSF1A [38]. The proteosomal degradation of the aforementioned proteins was abolished by the treatment with proteosomal inhibitors [38]. Conversely, siRNA-induced knockdown of *SKP2* led to growth restraint of HuH7 human HCC cells (expressing high SKP2 levels), which was paralleled by increase in the levels of $P21^{WAF1}$, $P27^{KIP1}$, $P57^{KIP2}$, P130, FOXO1, and RASSF1A proteins [38]. Thus, these findings suggest that the SKP2-mediated degradation of RASSF1A plays an important role in cell proliferation and survival. Finally, it has been found that the connector enhancer of KSR 1 (*CNK1*) gene, which interacts with RASSF1A and augments RASSF1A-induced cell death [39], is also often epigenetically silenced in human HCC [25].

Altogether, the present findings indicate that multiple mechanisms might play a role in RASSF1A inactivation in human HCC, further substantiating the need of RASSF1A silencing for liver cancer development and progression.

As concerns RASSF2, RASSF4, and RASSF5 isoforms (NORE1A and NORE1B), promoter hypermethylation seems to be the prominent mechanism responsible for their inactivation in liver cancer [6, 25, 27]. Indeed, epigenetic silencing of *RASSF2*, *RASSF4*, and *RASSF5* genes was inversely associated with low mRNA levels of the same genes [6, 25, 27].

4. Role of RASSF Proteins in Liver Cancer

Different from other tumor types, the biologic role of RASSF1A has been only minimally investigated in human liver cancer. Some interesting hints on the functional consequences of RASSF1A and NORE1A inactivation on hepatocarcinogenesis were obtained by analyzing a large collection of human HCC specimens [6]. In the latter samples, HCC displayed significantly lower levels of RASSF1A/H-Ras complexes compared with normal livers, indicating that the ability of RASSF1A to bind H-Ras is impaired in HCC. In contrast, RASSF1A/H-Ras complexes were increased in nontumorous surrounding livers, showing that RASSF1A is efficiently bound to H-Ras, thus presumably inhibiting H-Ras activity, at the preneoplastic stage. Furthermore, RASSF1A/NORE1A complexes were found only in the surrounding livers but not in the normal livers or HCC [6]. Because RASSF1A induces apoptosis through heterodimerization with NORE1A [40], these data indicate that RASSF1A-mediated cell death is abrogated in human HCC. Since the induction of RASSF1A and NORE1A leads to activation of MST1 and MST2 proteins [40], the levels of activated MST1 and MST2 were assessed. MST1 and MST2 proteins were phosphorylated in all surrounding nonneoplastic livers in association with caspase 3 cleavage [6]. Accordingly, protein levels of activated SEK1/MMK4-JNK and p38MAPK were low or absent in HCCs without MST1 and MST2 phosphorylation [6], consistent with the notion that MST1 and MST2 are upstream inducers of JNK and p38 MAPK proapoptotic pathways. Besides inducing apoptosis, MST1 and MST2 are crucial regulators of the Hippo signaling pathway. The latter is a conserved signalling cascade involved in the regulation of organ growth in *Drosophila* and vertebrates. In this cascade, MST1 and MST2 form a kinase cascade that is able to phosphorylate at the Ser127 residue the YAP oncoprotein, involved in unconstrained liver growth, leading to YAP inactivation [41–44]. The importance of the Hippo pathway in preventing hepatocarcinogenesis is underscored by the observations that disruption of the Hippo cascade associated with YAP activation triggers liver cancer development in the mouse. Indeed, liver-specific ablation of mouse *WW45* (homolog of the human *SAV1*) gene, an adaptor for the MST kinases, led to increased liver size and expansion of hepatic oval cells and, eventually, liver cancer development [45]. A similar growth effect and the unconstrained expansion of progenitor cells in the mouse liver resulted either from the combined deletion of MST1 and MST2 kinases [46–48] or overexpression of the YAP oncoprotein [49, 50]. Thus, due to its role as a positive regulator of the MST1 and MST2 kinases [11–15] and as inhibitor of MST1 and MST2 dephosphorylation [51], RASSF1A might play a crucial role in preventing liver malignant transformation.

In a recent investigation, transfection of the wild-type form of *RASSF1A* in the QGY-7703 human HCC cell line (expressing low levels of RASSF1A) resulted in fewer and smaller clones, decreased xenograft tumor volume and weight, and led to G1/S arrest both *in vitro* and *in vivo* when compared with cells transfected with the empty plasmid [52]. At the molecular level, transfection of wild-type *RASSF1A* resulted in decreased protein levels of cyclin D1. In addition, forced overexpression of wild-type *RASSF1A* triggered cell growth inhibition and increase in the percentage of cells in the sub-G1 phase following the treatment with mitomycin [52]. A novel proapoptotic pathway connecting RASSF1A to Bax via the Bax binding protein, modulator of apoptosis-1 (MOAP-1), has been described [53]. In this pathway, RASSF1A and MOAP-1 interact directly, and RASSF1A can activate Bax via MOAP-1, thus inducing cell death [53]. Of note, this pathway is impaired in most human liver cancer specimens, due both to inhibition of RASSF1A [53] and epigenetic silencing of *MOAP-1* (Calvisi et al., unpublished results), indicating that loss of RASSF1A-driven apoptosis might be an important molecular event in hepatocarcinogenesis.

Taken together, these data indicate that RASSF1A might exert its tumor suppressive activity on malignant hepatocytes by both inhibiting proliferation and stimulating apoptosis.

The role(s) of NORE1A and NORE1B have also been studied in human HCC. As concerns NORE1B, a microarray study was performed to identify its putative targets in the HEK-293T renal cell line [53]. A series of transcriptional alterations due to NORE1A induction were observed. Among the genes that showed some of the strongest changes in the microarray assay were eukaryotic translation elongation factor 2 (*EEF2*) and spermidine/spermine N1-acetyltransferase 1 (*SAT1*), whose levels were suppressed following *NORE1A* overexpression, and $p21^{CIP1}$, which was instead upregulated [54]. Further analysis showed that, in human HCC samples, *NORE1A* gene expression directly correlated with $p21^{CIP1}$ and inversely correlated with *EEF2* and *SAT1* expression [54]. EEF2 is a translation factor that mediates ribosomal translocation during peptide chain elongation and is activated by mitogenic stimuli [55]. *EEF2* is overexpressed in many tumor types and seems to play an important role in rendering tumor cells resistant to the translation-suppressing effects of hypoxia [56]. SAT1 is instead a spermidine kinase that plays a key function in the regulation of the intracellular levels of polyamines [57]. Polyamines play an important role in neoplastic growth, and polyamine synthesis inhibitors are of interest as chemopreventive agents [58]. Microarray analysis has been also performed to determine the signaling profile of RASSF1A in nonsmall cell lung cancer and neuroblastoma [59]. Noticeably, although RASSF1A sequence is 50% identical to

that of NORE1A, the two proteins promoted quite different alterations in gene expression [54, 59]. Indeed, SAT1 was the only target identified by both RASSF1A and NORE1A, thus confirming the hypothesis that the functions of NORE1A and RASSF1A are likely to be quite distinct. Several other upregulated targets following NORE1A overexpression that were identified in the array have also been associated with promotion of cell death and induction of growth suppression [54]. Among them, *BTG3* is a putative tumor suppressor gene and a target of p53 [60], whereas *PDCD2* has been implicated in apoptosis and proliferation control [61]. Thus, NORE1A promotes a number of alterations in transcription that might be involved in the repression of transformation. Nevertheless, the most interesting effect of NORE1A that was identified in the study was the upregulation of the $p21^{CIP1}$ tumor suppressor gene [54]. The induction of $p21^{CIP1}$ by NORE1A might explain the ability of NORE1A to induce G1 cell cycle arrest [62], since $p21^{\mathrm{CIP1}}$ has been shown to block the cell cycle at G1 by inhibiting cdk2 [63]. As mentioned above, the examination of a panel of human HCC showed that loss of NORE1A expression correlated closely with downregulation of $p21^{CIP1}$ expression [54]. These findings further substantiate the existence of a physiologic link between NORE1A and $p21^{\mathrm{CIP1}}$ in liver cancer. Moreover, it was demonstrated that NORE1A could only activate $p21^{\mathrm{CIP1}}$ in a wild-type *p53*-harboring tumor cell line, suggesting the requirement of a nonmutated *p53* gene for the transcriptional induction of $p21^{\mathrm{CIP1}}$ mediated by NORE1A [54]. Thus, the data indicate that NORE1A is involved in the modulation of one of the major human tumor suppressor pathways. This conclusion was further supported by the observation that mutations of *p53* gene and the inactivation of NORE1A were mutually exclusive events in human HCC [54]. The molecular mechanism(s) by which NORE1A can modulate p53 activity remains unknown. However, an increase of p53 in the nuclear compartment accompanied transfection of *NORE1A* in HuH6 human HCC cells in the same investigation. Thus, it is tempting to hypothesize that NORE1A promotes the nuclear localization of p53 via some posttranslational modification, such as phosphorylation or acetylation.

The role of NORE1B in liver cancer was also investigated [64]. In hepatocyte and hepatoma cell lines, *NORE1B*, *NORE1A*, and *RASSF1A* overexpression led to increase the percentage of cells in G0-G1 at the expense of the S-phase fraction [64]. Furthermore, *NORE1B* and *RASSF1A* insertion in hepatocyte lines resulted in an additional increase in the G2-M fraction, with consequent delay of cell cycle progression and suppression of cell growth. The molecular mechanisms whereby NORE1B reduces the cells in S-phase fraction have not been identified, although the SARAH domain and, to some extent, the RA domain of NORE1B were shown to be essential for growth suppression [64]. Another important discovery was that NORE1B antagonized c-Myc/Ha-Ras-induced transformation of embryonal cells [64]. Of note, *RASSF1A* alone was unable to antagonize cell transformation but enhanced greatly the *NORE1B* effect, which indicates cooperation of these genes. In accordance with the latter finding, the authors found that the NORE1B protein interacts closely with RASSF1A, as determined with fluorescence resonance energy transfer [64]. In further experiments, cell cycle delay by *NORE1B* overexpression was equally effective in hepatocyte cell lines with wild-type or mutant *Ras*, suggesting that NORE1B does not interact with Ras in order to exert its tumor suppressive function [64].

5. RASSF Proteins in Experimental Hepatocarcinogenesis

Few studies have investigated the status of RASSF proteins in experimental models of hepatocarcinogenesis to date.

The DNA methylation patterns of *Rassf1a* gene were investigated in the early phase of rat hepatocarcinogenesis induced by a choline-deficient L-amino acid-defined (CDAA) diet [65]. The livers of rats fed the CDAA diet for 4 and 8 days and 3 weeks were methylated in the *Rassf1a* gene promoter, while normal livers were all unmethylated. These results indicate that gene-specific DNA methylation patterns were found in livers of rats after short-term feeding of the CDAA diet, suggesting that gene-specific hypermethylation might be involved in the early phase of rat hepatocarcinogenesis induced by the CDAA diet [65].

The role of cell-cycle-regulating proteins, including Rassf1a, has been evaluated in preneoplastic lesions, dysplastic nodules, and HCC, chemically induced in genetically susceptible Fisher 344 (F344) and resistant Brown Norway (BN) rats [66]. Rassf1a protein levels exhibited no change or low increase in the lesions of F344 rats and consistent rise in dysplastic nodules and HCC of BN rats. Increase in Cks1-SKP2 ligase and proteosomal degradation of cell cycle regulators, including Rassf1a, occurred in F344 but not in BN rat lesions, indicating that posttranslational modifications of cell cycle regulators are under genetic control and contribute to determine a phenotype susceptible to HCC [66]. Furthermore, a gradual increase of Rassf1a/Nore1a/Mst1-driven apoptosis was detected in both rat strains, with highest levels in BN HCC, resulting in significantly higher apoptosis in BN than F344 HCC [67]. Taken together, these data indicate a control of the proapoptotic Rassf1a/Nore1A pathway by HCC susceptibility genes.

In another study, the underlying molecular events associated with tumor-promoting activity of 2-acetylaminofluorene (2-AAF), a complete genotoxic rat hepatocarcinogen, were investigated [68]. The results demonstrate that epigenetic alterations were responsible for driving hepatocarcinogenesis in this model. In particular, preneoplastic and neoplastic liver lesions exhibited increased histone H3 lysine 9 and histone H3 lysine 27 trimethylation in the promoter regions of *Rassf1a*, *p16INK4a*, *Socs1*, *Cdh1*, and *Cx26* tumor suppressor genes, early *Rassf1a* and *p16INK4a* promoter CpG island hypermethylation, and altered microRNA expression in preneoplastic livers of rats exposed to 2-AAF [68]. These changes were accompanied by dysregulation of the balance between cell proliferation and apoptosis, a fundamental protumorigenic event in hepatocarcinogenesis [67].

Altogether, these studies showed the frequent inactivation of Rassf1a either alone or in combination with Nore1a in rat models of hepatocarcinogenesis, implying a universal role of inactivation at least some of the RASSF proteins in liver malignant transformation and tumor progression. Based on these data, it appeared therefore surprising that *Rassf1a* null mice were tumor-prone and spontaneously developed a variety of cancer types, but no HCC or other liver tumors [69]. The lack of HCC development in *Rassf1a* knockout mice was unexpected and remains unclear. Presumably, hepatocarcinogenesis is not triggered by *Rassf1a* inactivation alone, but additional cellular (growth stimuli such as liver regeneration) and molecular (oncogene overexpression, loss of additional tumor suppressor genes) events are required for HCC development in *Rassf1a* knockout mice. A similar situation has been described, for instance, in *Sprouty 2* (a cellular inhibitor of the MAPK pathway) knockout mice. Indeed, inactivation of *Sprouty 2* by overexpression of its dominant negative form in the liver via hydrodynamic transfection was unable to induce significant changes in hepatocytes, whereas the coexpression of the *c-Met* protooncogene resulted in accelerated hepatocarcinogenesis in *Sprouty 2* deficient mice [70]. Therefore, it is plausible that additional cellular and/or molecular stimuli might be necessary for HCC development in *Rassf1a* knockout mice.

6. Concluding Remarks

A downregulation of RASSF1A, RASSF2, NORE1A, and NORE1B proteins has been described in human liver cancer. In particular, RASSF1A inactivation is a ubiquitous event and seems to be required for early and late steps of hepatocarcinogenesis, whereas silencing of NORE1A is associated with tumor aggressiveness. Some of the molecular mechanisms whereby RASSF1A, NORE1A, and NORE1B exert their tumor suppressive function have been determined, but presumably these proteins play many other roles in the control of hepatocytes proliferation and survival. In this regard, the study of the crosstalk between the RASSF proteins and the Hippo pathway will presumably provide important insights on liver cancer pathogenesis. A role in hepatocarcinogenesis might be also played by the newly discovered members of the RASSF family, known as N-Terminal RASSF proteins (RASSF7-RASSF10) [71], whose investigation has just begun. The use of appropriate genetically modified models will be highly helpful for the identification and dissection of the RASSF-mediated mechanisms as well as to test therapeutic approaches aimed at reactivating RASSF proteins and/or inactivating RASSF inhibitors, such as the SKP2 ubiquitin ligase, for the treatment of human liver cancer.

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Research Article

Combined *RASSF1A* and *RASSF2A* Promoter Methylation Analysis as Diagnostic Biomarker for Bladder Cancer

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Promoter hypermethylation, a widely studied epigenetic event known to influence gene expression levels, has been proposed as a potential biomarker in multiple types of cancer. Clinical diagnostic biomarkers are needed for reliable prediction of bladder cancer recurrence. In this paper, DNA promoter methylation of five C-terminal Ras-association family members (*RASSF1A, RASSF2A, RASSF4, RASSF5*, and *RASSF6*) was studied in 64 formalin-fixed paraffin-embedded (FFPE) bladder cancer and normal adjacent tissues using methylation-specific high-resolution melting (MS-HRM) analysis. Results showed that 73% (30/41) of transitional cell carcinoma, 100% (3/3) of squamous cell carcinoma, and 100% (4/4) of small cell carcinoma demonstrated promoter methylation of the *RASSF1A* or *RASSF2A* gene, but only 6% (1/16) of normal tissues had promoter methylation of *RASSF* genes. Testing positive for hypermethylation of *RASSF1A* or *RASSF2A* promoter provided 77% sensitivity and 94% specificity for identification of cancer tissues with an area under the curve of 0.854, suggesting that promoter methylation analysis of *RASSF1A* and *RASSF2A* genes has potential for use as a recurrence biomarker for bladder cancer patients.

1. Introduction

In 2011, about 52,000 men and 17,000 women will be diagnosed with bladder cancer in the United States. Before a normal cell transforms into a bladder cancer cell, a series of molecular alterations are accumulated to initiate the process of transformation. Although we do not fully understand the mechanisms, DNA alterations including hypermethylation and somatic mutation are commonly observed events in human cancer. In a recent bladder cancer study *FGFR3* mutation in combination with *APC*, *RASSF1A*, and *SFRP2* methylation markers provided a sensitivity of 90% using tissue samples and 62% using paired urine samples to identify the presence of cancer with 100% specificity [1]. In nonsmall cell lung cancer (NSCLC) and breast cancer, studies showed that *RASSF1A* had different frequencies of methylation depending on histology [2, 3]. In nasopharyngeal carcinoma, *RASSF2A* was frequently inactivated by its promoter methylation and the methylation correlated with lymph node metastasis [4]. The Ras-association family, also called *RASSF* tumor suppressor genes, currently includes 10 members. All of the *RASSF* proteins contain a Ras-association domain on their C-terminus (*RASSF 1–6*) or N-terminus (*RASSF 7–10*). Two important issues that are not previously addressed by studies of *RASSF* gene methylation are (1) whether all of the *RASSF* family members show aberrant methylation in bladder cancer and (2) whether methylation pattern of *RASSF* genes can be used as a diagnostic biomarker.

RASSF1A (Ras-association domain family 1 isoform A) is the first identified *RASSF* family member which is frequently epigenetically inactivated in a wide range of cancer types. As a tumor suppressor gene, *RASSF1A* regulates the activation of cell death [5], cell cycle [6], and microtubule formation [7]. The methylation signature of *RASSF1A* is thought to be among the earliest cellular changes in tumorigenesis [8]. As a potential tumor suppressor, *RASSF2* plays a role in apoptosis and cell cycle arrest and is frequently downregulated in lung tumor cell lines by hypermethylation [9]. Although the 5′ CpG island of *RASSF3* has been identified earlier, *RASSF3* does not show methylation in glioma tumor cell lines [10].

RASSF4 is broadly expressed in different human tissues, but its expression is down-regulated by promoter hypermethylation in a majority of tumor cell lines and primary tumors [11]. As a proapoptotic Ras effector, *RASSF5* (*NORE1A*) is frequently inactivated by promoter methylation in human tumors like glioma tumor cell lines, colorectal tumors, and lung cancer [12–15]. *RASSF6* promotes apoptosis by cooperating with activated *K-Ras* to induce cell death and inhibit the tumor cell survival [16]. A high frequency of *RASSF6* methylation is present in leukaemia-related diseases [17]. It appears that all of C-terminal *RASSF* family members have hypermethylation-induced gene inactivation in various types of cancer. While there is extensive literature on *RASSF1A*, other *RASSF* family members have not been studied as widely.

High-resolution melting (HRM) analysis is a new methodology that monitors the melting behavior of PCR amplicons by using DNA intercalating fluorescent dye [18]. Originally the LCGreen was used to develop a closed-tube method for genotyping and mutation scanning [19]. New high sensitive dyes such as EVA Green and SYTO 9 can be used at saturation concentration to monitor the denaturing process of PCR amplicons. Compared to traditional methylation specific PCR (MSP) method, HRM is a reliable and simple method for DNA methylation detection [20, 21].

In this study, to examine diagnostic value of *RASSF* gene methylation, we identified the methylation status of CpG islands associated with C-terminal *RASSF 1–6* in a group of formalin fixed paraffin embedded bladder cancer samples using a methylation specific HRM assay.

2. Methods

2.1. Control and FFPE Tumor Samples. Universal methylated and unmethylated DNA samples (Zymo Research Corp, orange, CA) were used as 100% and 0% methylated control. The methylated DNA was serially diluted in unmethylated DNA to create standard dilutions of 0%, 10%, 50%, and 100% methylated DNA. The standard dilutions from 100% to 0% were used to semiquantitatively measure promoter methylation status of C-terminal *RASSF* genes in FFPE samples.

FFPE blocks from 48 bladder cancer patients were collected by the department of pathology and the Human Tissue Resource Network at The Ohio State University. The study was conducted in accordance with the Institutional Review Board guidelines. We obtained 16 paired tumor and matched normal adjacent tissues and 32 tumor tissues (35 males and 13 females, male-to-female ratio 2.7 : 1; median age 67 years, range 28–90 years). Among these patients, 41 cases were diagnosed with transitional cell carcinoma; 4 cases were small cell carcinoma and 3 cases were squamous cell carcinoma. Clinicopathologic and demographic characteristic of bladder cancer samples are shown in Table 1.

2.2. DNA Extraction. DNA samples were extracted using Recover All Total Nucleic Acid Isolation Kit (Life Technologies Corporation, Carlsbad, CA). Briefly, 5–10 mg samples were sliced from paraffin blocks and deparaffinizated by xylene at 50°C, followed by 100% ethanol wash. The air-dry tissue samples were digested by proteinase K for 24 hrs in a microtube shaking incubator set at 50°C. The digested samples were mixed with appropriate volume of isolation additive and 100% ethanol. After passing the mixture through the filter cartridge, the DNA and RNA were retained on the filter. The RNA was removed by on-filter RNase digestion. The DNA was purified by washing buffer and eluted with 95°C nuclease-free water.

2.3. Bisulfite Modification. The FFPE DNA bisulfite modification was processed using EZ DNA Methylation Kit (Zymo Research Corp, Orange, CA). The double-stranded DNA was denatured in M-Dilution Buffer for 15 minutes at 37°C, and then CT Conversion Reagent was added to each sample. The samples were incubated in the dark at 50°C for 12 hours followed by 4°C for 10 minutes. After mixing with M-Binding Buffer, the samples were passed through a Zymo-Spin IC Column. The DNA purification and desulphonation were performed on the column. Finally, the bisulfite-modified DNA was eluted by M-Elution Buffer from column matrix.

2.4. MS-HRM Primer Design. MS-HRM is based on PCR amplification of bisulfite modified genomic DNA with subsequent HRM analysis of PCR amplicons. The primers were designed to amplify both methylated and unmethylated DNA. Because of DNA degradation in FFPE samples, the sizes of amplicons were limited to 80–180 bp. The free online tool from MethPrimer (http://www.urogene.org/methprimer/index1.html) was used specifically for primer design in this *RASSF* promoter methylation study. Primer sequences and amplicon lengths are shown in Table 2.

2.5. High-Resolution Melting Analysis (HRM). PCR amplification and high-resolution melting analysis were carried out sequentially on a CFX96 real-time PCR system (Biorad, Hercules, CA). PCR was performed in a 20 μL total volume containing: 10 μL 2X Type-it HRM PCR Master Mix (QIAGEN, Hilden, Germany), 1 μL 10 picomol/μL MS-HRM primer, 8 μL nuclease-free water, and 1 μL bisulfite converted DNA (theoretical concentration 10 ng/μL). The amplification consisted of 10 min at 95°C, followed by 40 cycles of 10 s at 95°C, 30 s at annealing temperature and 10 s at 72°C. High-resolution melting analysis were performed at the temperature ramping from 70–95°C by 0.2°C/s and florescence acquisition was set per manufacturer's recommendation.

2.6. Statistical Analysis. Methylation was classified as positive if at least 10% methylation was measured. The chi-square test was used to examine the significant differences of methylation depending on histology and staging ($P \leq 0.05$). To evaluate sensitivity and specificity of methylation as a predictive marker receiver operating characteristic (ROC) analysis was used.

Table 1: Patient demographic information.

	Study populations Total ($N = 64$)	Bladder cancer tissue specimen ($n = 48$)	Matched bladder cancer and normal adjacent tissues ($n = 16$)
Age (years): mean (range)	68 (28–90)	67 (28–90)	69 (53–90)
Gender			
Male	35	35	12
Female	13	13	4
Histologic cell type			
Transitional cell carcinoma	41	41	12
Small cell carcinoma	4	4	1
Squamous cell carcinoma	3	3	3
T stage			
T1	9	9	2
T2	11	11	3
T3	18	18	8
T4	6	6	1
Tx	4	4	4
N stage			
N0	27	27	9
N1	8	8	2
N2	8	8	3
Nx	5	5	2

3. Results and Discussion

To generate a profile of C-terminal *RASSF* gene epigenetic changes in bladder cancer, 64 bladder FFPE tissue samples were examined by methylation-specific HRM assay. The C-terminal RASSF family members (*RASSF1A*, *RASSF2A*, *RASSF4*, *RASSF5*, and *RASSF6*) were analyzed in this study. In the UCSC Genome Browser, we found a CpG island in the *RASSF3* promoter region. However, due to the dense CG dinucleotides on the CpG island of the identified *RASSF3* promoter, no appropriate HRM primers could be designed for this gene.

3.1. Quality Assessment of Methylation-Specific HRM Assay. HRM assay uses double-stranded DNA binding dyes and requires less PCR optimization than other methods. The principle of HRM depends on recording the melting profile of double-stranded DNA samples. As double-stranded DNA is denatured, the fluorescence signal from dye bound to double-stranded DNA decreases. The melting profile is related to amplicon length, DNA sequence and GC content. The high-resolution melting requires smaller temperature increase steps (<0.5°C/s) between each fluorescence reading, which can provide detailed information of amplicon melting behavior.

MS-HRM is a semi-quantitative method for rapidly assessing the presence of DNA methylation. The standard curve of methylation was used to confirm the *RASSF* gene methylation. The bisulfite-modified fully methylated DNA was diluted in bisulfite-modified fully unmethylated DNA to obtain a series of methylation percentage: 0%, 10%, 50%, and 100% methylation. Only samples containing more than 10% methylation were counted as methylated samples. The standard curve of *RASSF2A* methylation is shown in Figure 1.

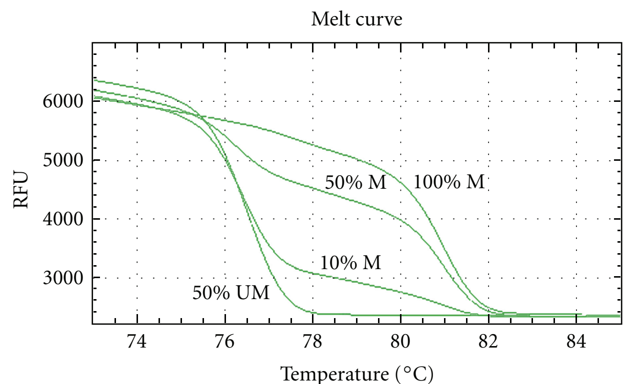

Figure 1: Standard curve constructed for *RASSF* promoter methylation. The dilutions of methylated DNA in unmethylated DNA are as follows: 0% methylation, 10% methylation, 50% methylation, and 100% methylation. Using Meth Primer software, a pair of primers was designed to amplify both methylated and unmethylated sequences after bisulfite conversion. The melting curves of 0% and 100% methylation indicate melting temperature of unmethylated sequence (76.6°C) and methylated sequence (81°C).

3.2. RASSF Family Member Methylation Profile in Tumor and Normal Adjacent Tissue Samples. Promoter methylation was analyzed in tumor and normal adjacent tissues from 16

Table 2: Primer sequences and melting temperature for *RASSF* genes.

Analysis	Sense primer	Antisense primer	Product size (bp)	T_{anneal} (°C)	Unmethylation T_{melt} (°C)	Methylation T_{melt} (°C)
RASSF1A	ATGTTAAGGGAATTTATTTAGAATGTATTT	AACCTTCACTTAAAATAAAAAAAA	146	53	73.2	75.6
RASSF2A	GAAGGGGTAGTTAAGGGGTAG	CCTCTACTCATCTATAACCCAAATAC	176	55	76.6	81
RASSF4	TAAATGGTTTGTGGTTTTTTGTTTT	AAAAACACCTTTATACAATCTAACC	175	55	72.8	74
RASSF5	GAAAGAGGTAGGGTTGAAGGTTTAG	TCACCTAAAACAACTACAAAATTCC	105	55	74	75.6
RASSF6	GTTTAGTTGAGTTATGTTTTGGGAG	AAAAAACCAATACCCTATCTCTACC	126	55	75.8	79.2

T_{anneal}, primer annealing temperature
T_{melt}, amplicon melting temperature.

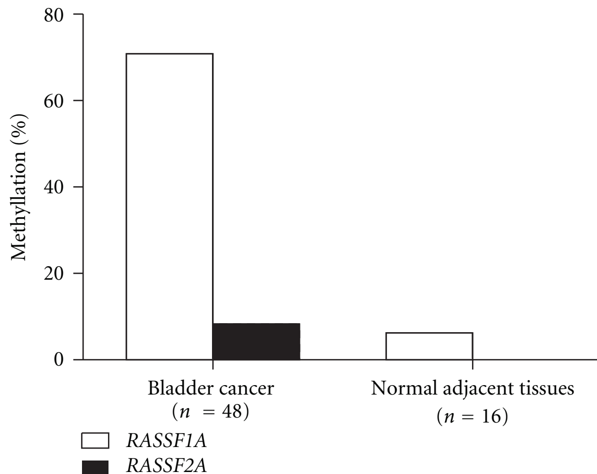

Figure 2: *RASSF1A* and *RASSF2A* methylation profiles of tumor and normal adjacent tissues. 71% (34/48) of tumor samples had *RASSF1A* promoter methylation, and 8% (4/48) had *RASSF2A* promoter methylation. No *RASSF4*, *RASSF5*, and *RASSF6* promoter methylation were detected.

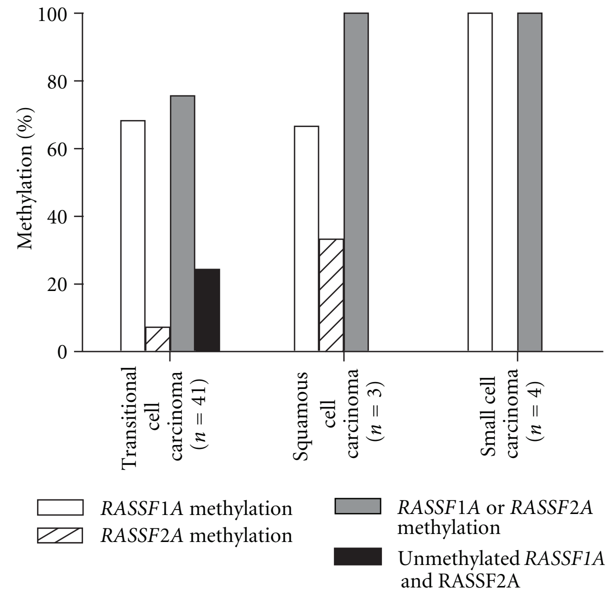

Figure 3: *RASSF1A* and *RASSF2A* methylation profiles of different histology. *RASSF1A* was methylated in 68% (28/41) of transitional cell carcinoma samples, in 100% (4/4) of small cell carcinoma and in 67% (2/3) of squamous cell carcinoma. *RASSF2A* was methylated in 7% (3/41) of transitional cell carcinoma samples, in 0% (0/4) of small cell carcinoma, and in 33% (1/3) of squamous cell carcinoma.

cases using MS-HRM [22]. 56% (9/16) of tumor samples were found to have *RASSF1A* promoter methylation, and 25% (4/16) of tumor samples showed *RASSF2A* promoter methylation, while only 6% (1/16) of the normal adjacent tissue samples showed *RASSF1A* promoter methylation and none of normal adjacent tissue samples showed *RASSF2A* methylation. *RASSF 4*, *5,* and *6* were not found to be methylated in either tumor or normal adjacent tissues. Figure 2 demonstrates that tumor and normal adjacent tissue showed different *RASSF1A* and *RASSF2A* methylation profiles.

Among the 48 patients with bladder cancer, *RASSF1A* promoter methylation alone had 71% sensitivity and 94% specificity and an area under the curve (AUC) of 0.823 to correctly identify bladder cancer tissue whereas *RASSF1A* and *RASSF2A* together had 77% sensitivity and 94% specificity and AUC of 0.854. *RASSF1A* and *RASSF2A* promoter methylation did discriminate bladder cancer tissue from normal adjacent tissue ($P < 0.0001$).

3.3. RASSF1A and RASSF2A Methylation Profile in Different Histological Samples. Patient characteristics are summarized in Table 1. 41 patients had tumors with transitional cell carcinoma features 4 with small cell carcinoma, and 3 with squamous cell carcinoma. *RASSF1A* was methylated in 68% (28/41) and *RASSF2A* in 7% (3/41) of transitional cell carcinoma samples. Only one sample had methylation of both the *RASSF1A* and *RASSF2A* promoters. *RASSF1A* was methylated in 100% (4/4) and *RASSF2A* in 0% (0/4) of small cell carcinoma. *RASSF1A* was methylated in 67% (2/3) and *RASSF2A* in 33% (1/3) of squamous cell carcinoma (Figure 3). The frequency of *RASSF1* and *RASSF2* promoter methylation together showed no significant difference with histology in our study ($P = 0.295$).

The reasons that lead to aberrant CpG island methylation of *RASSF1A* and *RASSF2A* in transitional cell carcinoma, small cell carcinoma, and squamous cell carcinoma of the bladder are not well understood. Recent data by Li et al. investigating nonsmall cell lung cancer showed that *RASSF1A* promoter region CpG islands were methylated in 55% of adenocarcinomas, 25% of large cell carcinomas, and 25% of squamous cell carcinomas [3]. This study indicates that promoter methylation of *RASSF* gene family members might be dependent on histology in nonsmall cell lung cancer.

3.4. RASSF1A and RASSF2A Methylation Profile at Different T Stages. 60 malignant bladder tumor and normal adjacent tissue samples from patients with different T stages (16 normal adjacent tissue samples, 9 stage T1, 11 stage T2, 18 stage T3, and 6 stage T4) were analyzed to detect the *RASSF* promoter methylation changes of bladder cancer patients. Methylated *RASSF1A* promoters were only found in 6% (1/16) of normal adjacent tissues. The percentage of promoter methylation positive patients increased with T stage, being lower in T1 tumors and higher with higher stage. The percentage of samples with methylated *RASSF1A* genes was 55% (5/9) in stage T1, 73% (8/11) in stage T2, 78% (14/18) in stage T3, and 83% (5/6) in specimens from T4 tumors (Figure 4). The frequency of *RASSF1* and *RASSF2* promoter methylation was not associated with T stage ($P = 0.363$). Due to lack of samples with *RASSF2A* methylation, no association between *RASSF2A* methylation and T stage was identified in the current data set. Similar results were found in a recent lung cancer study [23]. The *RASSF2A*

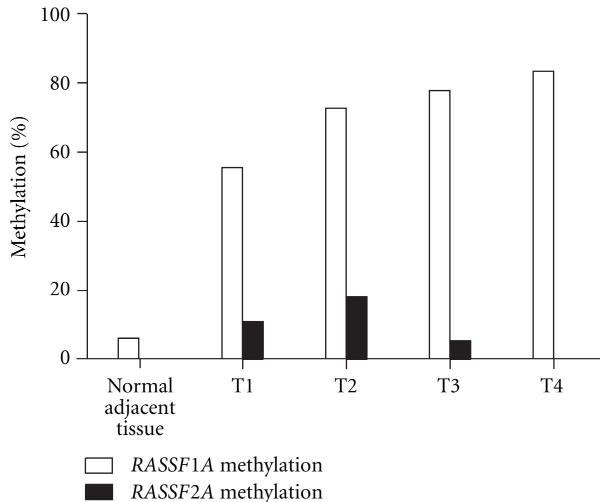

Figure 4: *RASSF1A* and *RASSF2A* methylation profiles in different T stage. *RASSF1A* was only 6% (1/16) in normal adjacent tissues, but percentage of tumor samples with methylated *RASSF1A* genes had a range of 55%−83% from T1 to T4 stage. The *RASSF2A* promoter had a low level (0–18%) at different T stages.

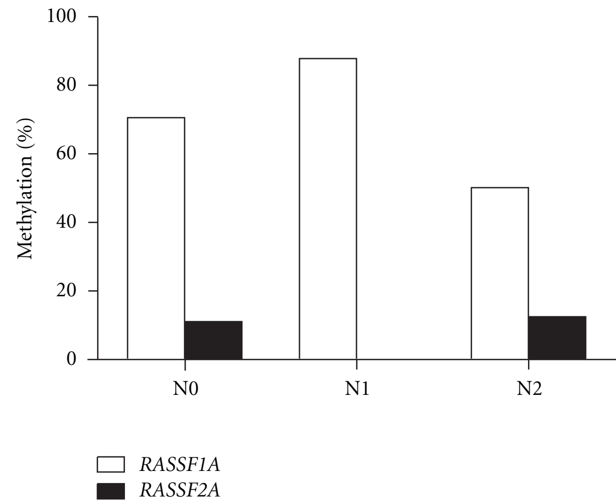

Figure 5: *RASSF1A* and *RASSF2A* methylation profiles in different N stages. Percentage of *RASSF1A* methylation had a range of 50–87.5% in different N stage, and percentage of *RASSF2A* had range of 0%−11%. Aberrant methylation of *RASSF1A* and *RASSF2A* promotor showed no relationship to lymph node metastasis.

promoter methylation was found at low levels (0–18%) at different T stages.

3.5. RASSF1A and RASSF2A Methylation Profile at Different N Stages. We examined methylation status of *RASSF1A* and *RASSF2A* in 27 bladder tumor samples with stage N0, 8 samples with stage N1, and 8 samples with stage N2. Percentage of *RASSF1A* promoter methylation had a range of 50–87.5% in tissues from patients with different lymph node metastasis stage. The percentage of samples with methylated *RASSF2A* was 11% (3/27) for stage N0, and 6% (1/16) for stage N1/2 (Figure 5). Based on the result of chi-square test, there is no significant difference between the frequency of *RASSF1* and *RASSF2* promoter methylation in lymph node positive and negative patients.

In nasopharyngeal carcinoma, aberrant methylation of *RASSF2A* promotor was found to be associated with lymph node metastasis [24]. Although both *RASSF1A* and *RASSF2* protein can function as a negative effector of Ras protein in tumor formation, *RASSF2A* and *RASSF1A* have apparently different functions in different type of tumors.

3.6. RASSF4, RASSF5, and RASSF5 Methylation. *RASSF1-6* share a variable N-terminal sequence followed by a Ras-association domain [25]. The HRM analysis showed no detectable promoter methylation of *RASSF4*, *RASSF5* and *RASSF6* in bladder cancer and normal adjacent tissue samples.

4. Discussion

DNA methylation and histone modification are widely studied epigenetic events. Promoter hypermethylation has been proposed as a potential diagnostic or prognostic biomarker in various cancers. Recent research showed that urine is potentially useful for bladder cancer screening [26, 27]. Methylation status of certain genes identified in urine samples showed higher sensitivity than the conventional urine cytology method. These studies indicated that detection of promoter methylation in urine specimen could potentially provide a simple, noninvasive, and sufficiently sensitive method for bladder cancer screening in the future.

In our study, a new methodology, methylation-specific-high resolution melting analysis was used to examine the melting behavior of methylated or unmethylated *RASSF* gene amplicons. This provides a simple and reproducible method for promoter methylation assessment. We studied DNA promoter methylation of five *RASSF* family members (*RASSF1A*, *RASSF2A*, *RASSF4*, *RASSF5*, and *RASSF6*) in FFPE bladder cancer tissues and normal adjacent tissues. We identified distinctive *RASSF1A* and *RASSF2A* gene promoter methylation profiles that differentiate between bladder cancer and normal adjacent tissue samples. Using *RASSF1A* and *RASSF2A* genes together showed an acceptable sensitivity (77%) and high specificity (94%) identifying bladder cancer tissues. Previous studies have identified *RASSF1A* promoter methylation as a potentially useful urine biomarker for the presence of invasive bladder cancer [26, 28, 29]. We now show that the addition of *RASSF2A* promoter methylation analysis can improve the sensitivity potentially without compromising specificity. There was no significant correlation of *RASSF1A* methylation with histology and N stage. As discussed by Serizawa [1], results also showed that *FGFR3* mutation in bladder cancer when combined with methylation markers (*APC*, *RASSF1A* and *SFRP2*) provided a sensitivity of 90% to identify bladder tumors. It remains to be shown if the addition of an *RASSF2A* promoter methylation assay to the previously published urine biomarker assay can indeed improve sensitivity when using urine samples. Limitations of the study include small sample size or lack

of a validation data and sample set. In addition we did not have sufficient data to analyze if *RASSF* gene family promoter methylation could predict the recurrence of bladder cancer.

Methylation analysis of both *RASSF1A* and *RASSF2A* genes appeared to increase the sensitivity of discriminating cancer from normal adjacent tissue. The addition of *RASSF2A* methylation analysis to recent bladder cancer biomarker signatures has the potential to further increase sensitivity for bladder cancer diagnosis. *RASSF1A* and *RASSF2A* promoter methylation analysis could be useful as a biomarker to detect the presence of bladder cancer recurrence.

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