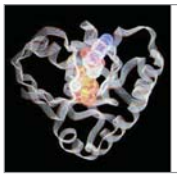

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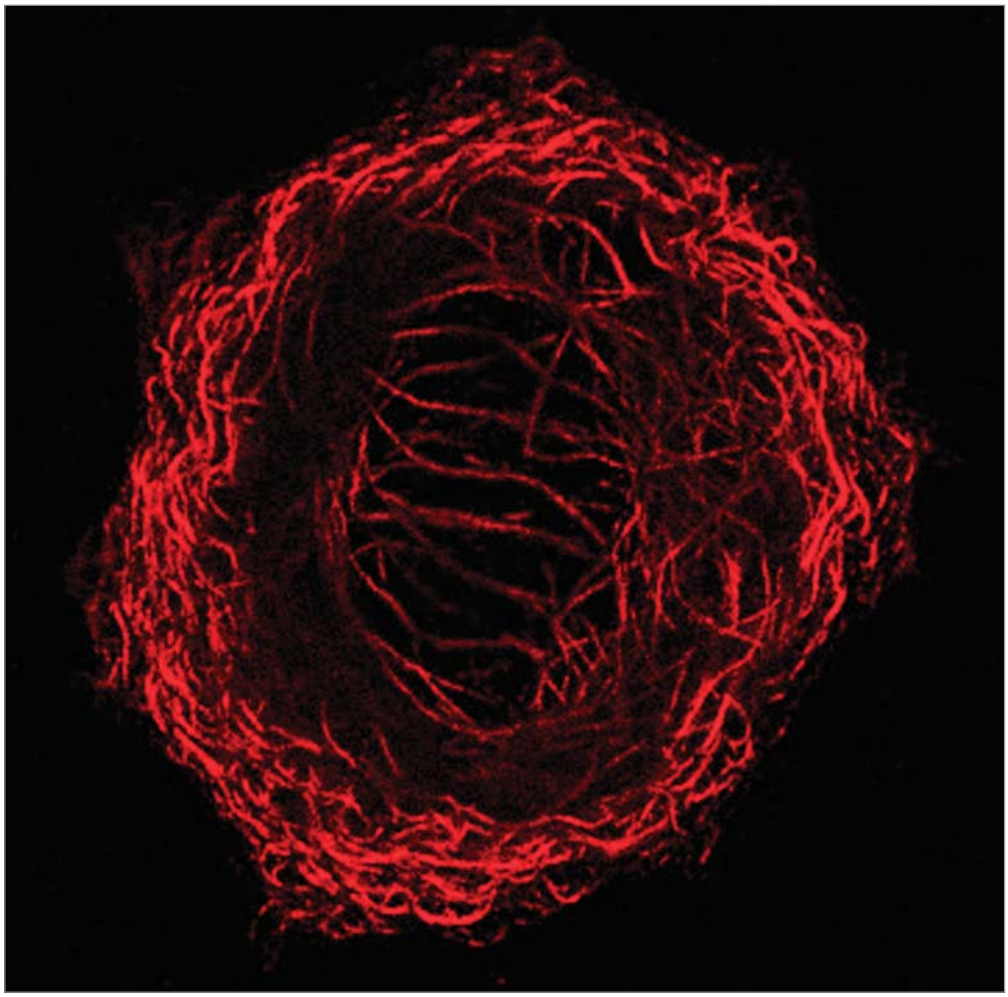

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RASSF1A

Not a prototypical Ras effector

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Key words: RASSF1A, apoptosis, Ras, tumor suppressor gene, 14-3-3, MOAP-1, microtubule, mitosis

Abbreviations: C19ORF5, chromosome 19 open reading frame 5; ERK, extracellular regulated kinase; ER, endoplasmic reticulum; GEF, guanine exchange factors; GAP, GTPase activating proteins; GRB2, growth factor receptor-bound protein 2; Nore, novel ras effector; PI3K, phosphatidylinositol-3-kinase; PKB, protein kinase B; PTPN, protein tyrosine phosphatase non-receptor type; RAC, ras-related C3 botulinum toxin substrate; RAPL, Regulator of adhesion and polarization enriched in lymphocytes; 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; SHC, Src homology 2 domain containing; SOS, son of sevenless

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The Ras association domain family (RASSF) of genes are commonly silenced by promoter specific methylation in human cancers. After the cloning of the first two family members in early 2000 (RASSF1 and RASSF5), eight other related genes have been identified (RASSF2, 3, 4 and 6–10). The unifying motif amongst all RASSF family members is the presence of the Ras association (RA) domain that could potentially associate with the Ras family of GTPases. Detailed analyses have determined that RASSF family members are tumor suppressor proteins, activators of cell death, cell cycle modulators, microtubule stabilizers and possibly inflammatory mediators linked to NFκB. As such, exploring the biological function of this gene family is needed and if indeed RASSF proteins could be the missing link between Ras signaling and apoptosis. Several RASSF family members have been demonstrated to associate with Ras. However, there is still controversy regarding the ability of RASSF1A to utilize Ras to promote cell death and of the importance of the RASSF1A RA domain. The focus of this review is to highlight the importance of Ras binding to the RASSF family of proteins and discuss what we currently know about the biology of RASSF1A.

A Short History of Ras Signaling

The Ras superfamily of proteins contains over 100 members that are GTP-regulated molecular switches for signaling pathways involving cellular processes as diverse as proliferation, migration, cell death and differentiation. The Ras signaling pathway has been the subject of intense investigation since it was first characterized in 1993.[1] Shortly after this discovery it was determined that this pathway is highly conserved in Drosophila, *S. cerevisiae* and *C. elegans*. Three main Ras GTPase isoforms exist in mammals that are commonly targeted for germline mutations in several cancers: H-, N-, K-Ras (isoforms 4A and 4B). Other prominent members of the Ras GTPase superfamily include Rap1 (involved in integrin signaling), Rho (involved in neuronal signaling) and the Rac family of GTPases (the latter of which have additional roles in cytoskeleton reorganization).[2] Activating mutations in the Ras oncogenes account for about 30% of germline mutations in human cancers. In addition, it is known that abnormal Ras signaling, resulting from genetic changes in Ras oncogenes and Ras upstream activators or downstream effectors, can contribute to the origin of about 70% of all human cancers. Therefore, an understanding of Ras signaling is of vital importance to the origin of cancer and in designing new targets for treating human malignancies.

Ras proteins are strategically positioned at the inner surface of the plasma membrane, at the endoplasmic reticulum (ER) and there is recent evidence for Ras activation at the golgi complex.[2] It is now well established that membrane association of Ras is a requirement for its biological activity regulated by membrane proximal events involving the molecular switch of Ras-GDP to Ras-GTP (**Fig. 1**). Extracellular signals promote the GTP loading of Ras[2] by recruiting and activating the Ras guanine exchange factors (GEFs). Reversal of

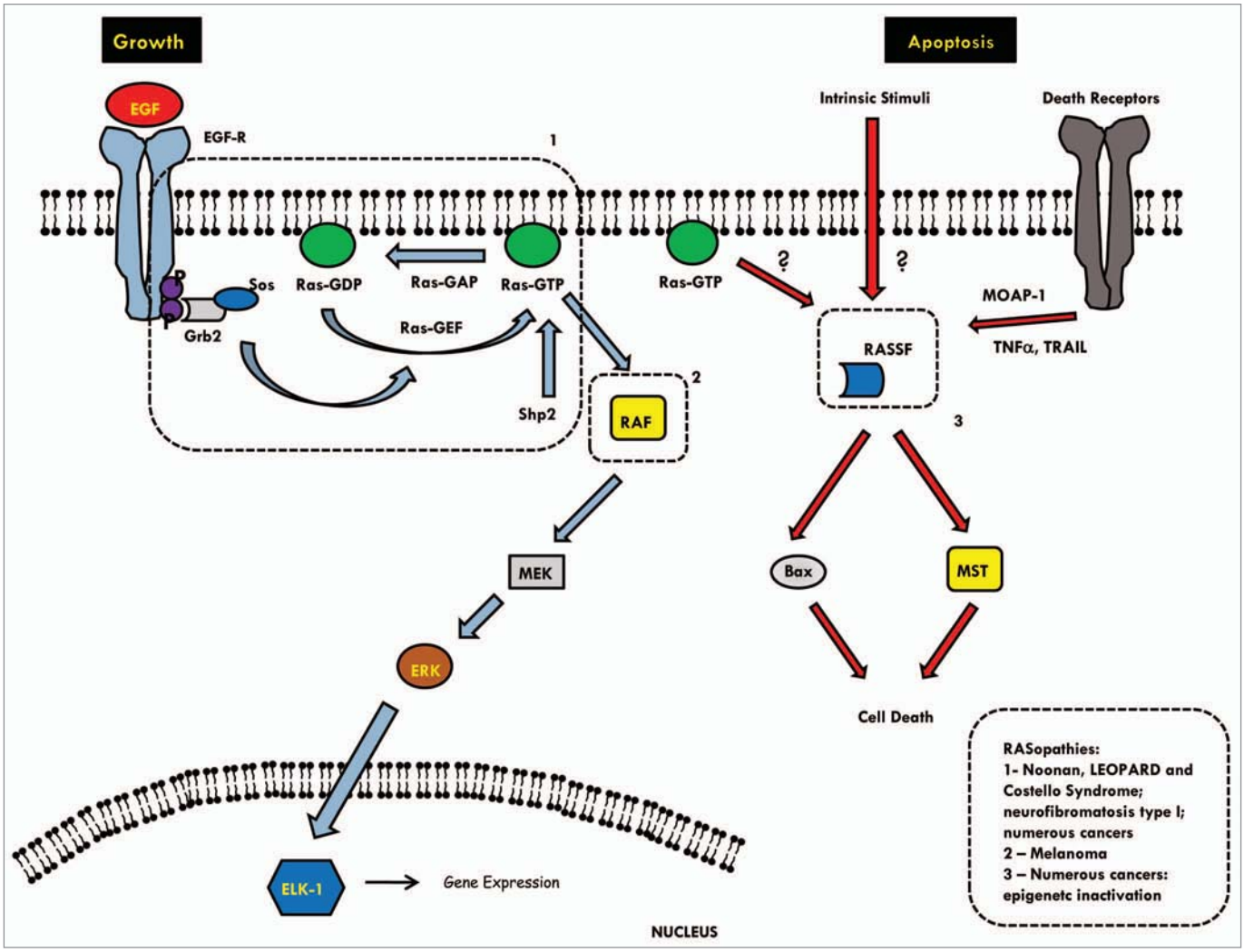

Figure 1. A simplified model of Ras signaling. Ras signaling pathways originate from growth factor binding and activation of its receptor such as the epidermal growth factor [EGF] binding to its receptor, EGF-R. The activation of the EGF-R by EGF primes the autophosphorylation of the cytoplasmic domain of EGF-R (P) and the recruitment of adapter proteins, Grb2 and Sos. Sos is a primary Ras-GEF that will promote GTP loading of Ras and subsequent Ras activation. Ras can then continue the signaling cascade to Raf-MAPKK (MEK)-MAPK (ERK) and eventually to the activation of the transcription factor Elk-1. Once activated by phosphorylation, Elk-1 can then acquire DNA binding competency to activate gene transcription to drive proliferation. Elk-1 is just one of many transcription factors modulated by Ras signaling and is only shown here for simplicity. There is evidence for Ras activation of cell death and it is thought to proceed through the RASSF family of proteins. In the bottom right corner of this figure is a limited list of diseases that are a direct result of abnormal Ras signaling (the RASopathies).

Ras activation is achieved by the actions of the Ras GTPase activating proteins (GAPs). GTP-Ras undergoes a conformational change in switch regions I and II in order to promote the association of downstream effectors.[2] Growth factor receptors such as the prototypical epidermal growth factor function primarily to promote this conformational change in Ras in order to initiate a signal cascade to the cytoplasm and eventually to the nucleus.

Growth factor stimulation results in the autophosphorylation of the cytoplasmic domain of these receptors that functions to prime the recruitment of signaling molecules Shc, Grb2 and the GEF Sos (**Fig. 1**). Sos functions to switch Ras-GDP to Ras-GTP and activated Ras can in turn activate the serine/threonine kinase, Raf, the first characterized downstream effector of Ras. Raf then activates downstream kinases such as the mitogen activated kinase kinase kinase (MAPKKK) to continue the signal cascade to mitogen activated kinase kinases (MEK1 and MEK2) and then to the MAP kinases, extracellular regulated kinases (p44/ERK 1 and p42/ERK2). ERKs then translocate to the nucleus to activate transcription factors such as Elk-1 allowing them to acquire DNA binding competency. Once activated, Elk-1 can drive transcription of genes important to growth and proliferation. Collectively, the components above are part of the well studied Ras-Raf-MAPK pathway, a highly conserved universal growth signaling pathway.

The RASopathies: diseases resulting from abnormal Ras signaling. The Ras-Raf-MAPK pathway functions as one of the main effector pathways of Ras to regulate growth. As such, this pathway is

also one of the primary targets for genetic changes resulting in the RASopathies, syndromes caused by abnormal Ras signaling.[2-4] These syndromes are well characterized as melanomas (B-Raf mutations and loss of RASSF1A expression), lung and pancreatic cancers (mutations in K-Ras and loss of RASSF1A expression), Noonan syndrome (mutations in PTPN11, K-Ras, SOS and Raf-1), LEOPARD syndrome (a syndrome similar to Noonan syndrome having mutations in PTPN11 and Raf-1), neurofibromatosis (NF) type 1 (mutations in the NF1, a Ras-GAP), Costello syndrome (mutations in H-Ras) to mention a few.[3,4] PTPN11 encodes the non-receptor protein tyrosine phosphatase, SHP-2, a phosphatase that is required for activation of Ras and other signaling cascades such as the Jak/Stat and insulin receptor pathways.[5] In addition, mutations in H-Ras and N-Ras are also very prevalent in numerous human cancers that can effectively promote uncontrolled Ras-dependent activation. As such, there has been and continues to be considerable interest in searching for a "drugable" target within the Ras-Raf-MAPK pathway that may suppress or inhibit the growth promoting effects of these mutations. To date, most efforts have failed to specifically target this pathway without toxic side effects. The major limiting factor has been target specificity for the tumor cell while avoiding the inhibition a functional Ras-Raf-MAPK pathway in surrounding normal cells. Ras function is complicated in that it is involved in numerous biological processes; regulated by multiple growth factors, GEFs and GAPs; activated in different membrane compartments (plasma membrane, ER, golgi complex) and by multiple isoforms of Raf, MEK and ERK leading to the activation of numerous transcription factors. The combined permutations of these variables produce very distinct Ras signaling pathways that, if dysregulated, can result in the occurrence of the RASopathies (**Fig. 1**).

Ras and Apoptosis: An Unresolved Debate

There is no debate as to the significant role that Ras plays in growth control and proliferation and how this is important for the appearance of malignancy. It is well documented that sustained activation of active Ras is required for tumor development and for tumor cells to avoid apoptosis.[6] Overexpression studies, the presence of Ras mutations and mouse genetic knockouts of Ras family members have all proven that Ras has a robust role in growth control. However, considerable debate remains as to the importance of Ras in cell death or apoptosis, a concept that is counter-intuitive to its role in promoting cellular growth. It has been reported that, depending on the cell type and environment, Ras can be anti-apoptotic or pro-apoptotic. There is speculation that in normal cells Ras may be upregulated to promote protective pro-apoptotic responses in order to prevent tumorigenesis. Ras can mediate an anti-apoptotic effect by the activation of phosphatidylinositol 3-kinase (PI3K) and Akt/PKB that will in turn phosphorylate Bad and sequester Bad to 14-3-3. Bad is a pro-apoptotic member of the Bcl-2 family of apoptotic regulators. This event will relieve the Bad inhibition of Bcl2 or Bcl-x_L resulting in an anti-apoptotic response.[7,8] In addition, the activation of Akt/PKB can also phosphorylate IκBα kinase (IKK) and subsequently activate NFκB to promote survival. This is mainly carried out by promoting the transcription of anti-apoptotic proteins such as inhibitor of apoptosis (IAPs). Therefore, Akt/PKB and NFκB pathways activated by Ras will promote survival and thus provides examples of how Ras can intersect with signaling pathways to provide an anti-apoptotic role.

Several reports suggest that upon growth factor withdrawal Ras can promote apoptosis via a Ras/Raf/MEK/ERK pathway.[8] This has been observed for IL-3 withdrawal in the pro-B cell BaF3 [9] and serum rich media withdrawal in 3Y1 rat fibroblasts.[10] Furthermore, in T lymphocytes, IL-2 or antigen receptor stimulation can activate Ras and drive cellular proliferation. However, if proper coreceptor stimulation does not occur, Ras activation can lead to apoptosis.[11] Furthermore, overexpression of activated Ras in T cells can lead to increased expression of Fas ligand and increased cell death most likely via autocrine stimulation of Fas ligand.[7,12] Thus, both excessive Ras activation and growth factor withdrawal can promote cell death in a Ras dependent manner. This may suggest that under conditions of abnormal homoeostasis, Ras functions in a "protective" manner to remove the cell by apoptosis in order to prevent the inheritance of abnormality. These examples illustrate the controversial nature of the influence of Ras signaling on apoptosis and how little is known about the molecular mechanisms that link Ras to cell death.

The RASSF family of proteins brought excitement to an aspect of Ras signaling that needed some answers, that is, the role of Ras in apoptosis. As mentioned earlier, the RASSF family of proteins have an RA domain that can potentially associate with Ras. However, unlike most Ras effectors, the RASSF family of proteins lack catalytic activity and function as adaptor proteins that can regulate multiple signaling pathways including two (and maybe more) apoptotic signaling pathways. The association of Ras with RASSF family of proteins suggested that we had finally found a Ras effector that required Ras association in order to promote apoptosis. Ten family members exist that contain an RA within the N-terminal (RASSF7-10) or C-terminal region (RASSF1-6) of the primary sequence.[13,14] RASSF5A was initially named novel Ras effector 1 (Nore1) and RASSF5B is a splice variant of RASSF5 that was independently identified as RAPL, regulator of adhesion and polarization enriched in lymphocytes.[15] RASSF9 was originally named P-CIP1, petidylglycine α-amidating monooxygenase [PAM] C-terminal interactor 1 as it associated with the C-terminus of PAM. Reports from numerous laboratories suggest that the RA may not be utilized equally by all RASSF family members. We have summarized the Ras association studies with RASSF family members in **Table 1**. What is revealing is that there are contradictory reports of Ras association with RASSF1A. If this association does occur it is with active K-Ras indirectly through heterodimerization with RASSF5A.[16] We have not observed RASSF1A association with N-, H-, K- or R-Ras association either with wild type or active mutants of these Ras proteins (unpublished observations). We support the studies by Ortiz-Vega et al. (2002) [16] suggesting that Ras

Table 1. Summary of what is known about Ras association with the RASSF family of proteins

RASSF family member	Associated RAS isoform	Cell type and condition	References
RASSF1A	• H-Ras G12V: only in the presence of RASSF5A/ Nore1A • K-Ras V12 association promotes RASSF1A/MOAP-1 association • K-Ras WT associates very weakly with RASSF1A and this was lost with the K-Ras V12/Y40C mutant • R-Ras can associate with RASSF1A	(1) COS-7 cells, overexpressed Flag-RASSF1A and GST HRasG12V (2) 293-T cells, overexpressed HA-RASSF1A, Myc-MOAP-1 and Ha-K-Ras 12V or HA-KRas 12V/Y40C	Vos et al. (2005)[40] Rodriguez-Vician et al. (2004)[17] Disputed in Ortiz-Vega et al. (2002)[16]
RASSF1C	H-Ras (G12V) but not with H-Ras (E37G) or Ras (C186S)	(1) 293-T cells, overexpressed RASSF1C and H-Ras G12V; (2) In vitro: MBP-1C (RA) and H-Ras (GTP) form	Vos et al. (2000)[56] Disputed in Ortz-Vega et al. (2002)[16]
RASSF2	K-Ras 4B (G12V)	293-T ells, overexpressed HAK Ras 4B (G12V) and Flag-RASSF2	Vos et al. (2000 and 2003)[56,23] Donninger et al. (2010)[57]
RASSF3, 7, 8, 10	Not tested	-	-
RASSF4	K-Ras G12V	293-T cells, overexpressed FLAG-RASSF4 with HA-K-Ras G12V	Eckfeld et al. (2004)[58]
RASSF5A/Nore1A	• H-Ras (G12V) >>> Ki-Ras • No H-Ras N17 association	COS-7, overexpressed Flag-RASSF5A and GST-H-Ras G12V	Ortiz-Vega et al. (2002)[16]
	• Ki-Ras, R-Ras, M-Ras, R-Ras3, Rap2A	Yeast-two hybrid	Ortiz-Vega et al. (2002)[16]
	H-Ras G12V	293-T and COS-7 cells, overexpressed RASSF5A and H-Ras G12V	Vost et al. (2003)[23] Stieglitz et al (2008)[22] Ortiz-Vega et al. (2002)[16]
	K-Ras G12V	Rassf5a$^{-/-}$/K-Ras G12V mice	Park et al. (2010)[24]
RASSF5B/RAPL	Rap1 G12V	Overexpressed EGFP-RASSF5B with FLAG-Rap1 G12V	Fujita et al. (2005)[15]
RASSF6	• K-Ras G12V • No association to K-Ras G12V/Y40C • Note: Association with K-Ras V12 promotes RASSF1A/MOAP-1 association	293-T cells, overexpressed FLAG-RASSF6 and HA K-Ras G12V	Allen et al. (2007)[59]
	• H-Ras G12V >> K-Ras G12V = MRas G12V >>> N-Ras G12V	COS-7 cells, overexpressed Ras isoforms were used in pull-down experiments with MBPRASSF6	Ikeda et al. (2007)[32]
RASSF9	N-Ras > K-Ras >> R-Ras	293-T cells with GST-Ras proteins co-transfected with myc-tagged RASSF9 RA domain	Rodriguez-Vician et al. (2004)[17]

Associations of RASSF family members with Ras oncogenes are indicated with a description of cell lines used in the referenced articles. Symbols of "=" or ">>" denote order of binding associations mainly based on immunoprecipitations studies as indicated.

association with RASSF1A is indirect and weak. In contrast, convincing data does exist for the ability of RASSF2, 4, 5 and 6 to associate with the active forms of H-Ras and K-Ras (**Table 1**).[17] For these associations, Ras proteins aid in the ability of the RASSF proteins to induce cell death, reduce colony formation (and thus growth stimulating potential) and reduce tumor formation in nude mice. RASSF9 is the only N-terminal RASSF family member that has been shown to associate with N-Ras, K-Ras and R-Ras by co-immunoprecipitation experiments.[17] The ability of the other N-terminal RASSF members to associate with Ras has yet to be tested.

RASSF1A: A complex tumor suppressor regulating apoptosis and cell cycle control. RASSF1A, the founding member of this family, has now been shown to be one of the most methylated genes in human cancers. Not only is it commonly silenced, but it is thought to be one of the earliest detectable changes in tumorigenesis. RASSF1A contains several domains within its primary sequence that may be important for its role as a tumor suppressor protein (**Fig. 2**). Some of these have been characterized and demonstrated to be important for death receptor association (the C1 zinc finger domain),[18] DNA damage repair of double strand breaks (the ATM phosphorylation site) [19] and for association with pro-apoptotic kinases MST1 and MST2 (the SARAH domain).[20] Although important for death receptor association, it is unclear if the potential diacylglycerol (DAG) C1 domain may play a role in membrane lipid binding similar to how the C1 domain of protein kinase C binds to membrane lipids. No function has been assigned to the potential SH3 binding PxxP motif on RASSF1A (**Fig. 2**).

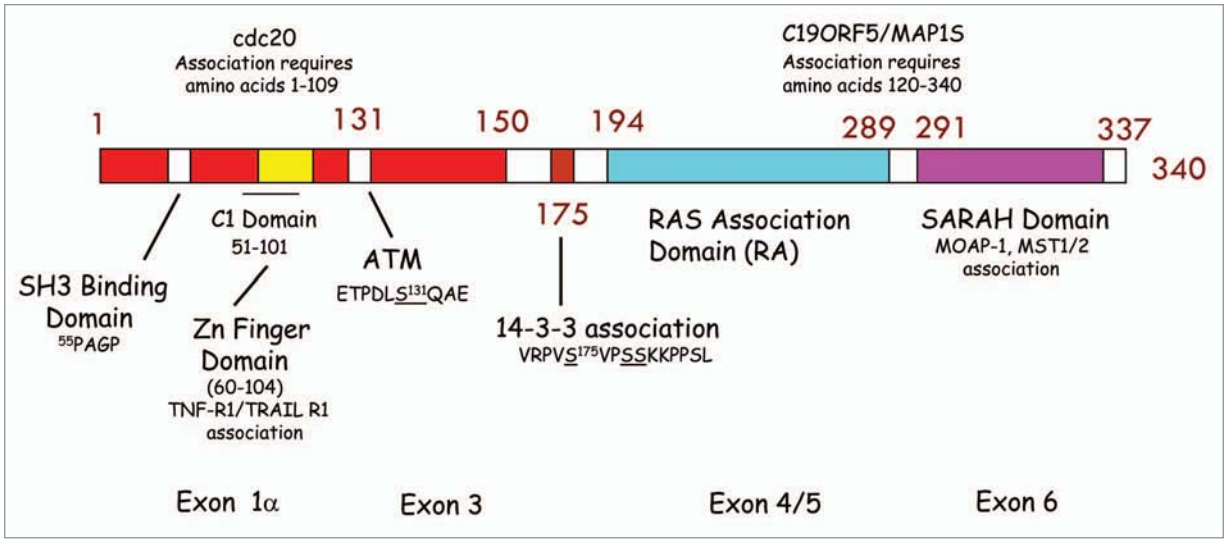

Figure 2. Schematic of RASSF1A. The SH3 binding domain of RASSF1A has the sequence PAGP that is conserved in mouse and human forms of RASSF1A and found as PQDP in RASSF5A. The ATM (Ataxia telangiectasia mutated) phosphorylation site is underlined with surrounding residues shown. ATM is a serine/threonine protein kinase that is a DNA damage sensor activated upon double strand breaks (DSBs), apoptosis or the addition of genotoxic stresses such as ultraviolet A light (UVA). The binding sites for several RASSF1A effector proteins are shown. The Ras association domain (RA) may potentially associate with the Ras family of oncogenes. The SARAH domain modulates heterotypic associations with the sterile-20 like kinases, MST1 and MST2. Approximate positions of exons and amino acids are also indicated as well as the identification of several polymorphisms to RASSF1A. Adapted from El-Kalla et al. (2010).[49] See text for details on the other functional domains.

The NMR solution structure of RASSF5A/Nore1A revealed a surprising intramolecular association between the C1 domain of RASSF5A and the RA.[21] Interestingly, Ras can disrupt this association and promote a more "open" conformation of RASSF5A.[21] We have modeled the C1 domain of RASSF1A based on the NMR structure of RASSF5A and found surprising similarity between the two.[18] RASSF5A, the closest family member, has 60% amino acid identity to RASSF1A.[14] We speculate that the C1 domain of RASSF1A may associate with the RA of RASSF1A in an intramolecular complex. However, since RASSF1A binds very weakly to Ras, we hypothesize that the RA of RASSF1A may associate with a yet unknown GTPase that may, in a similar manner to RASSF5A, displace the C1 domain to aid in further downstream signaling.

What is known about the RA of RASSF5A is that it is not sufficient to promote Ras association but may require an extended region spanning amino acids 203–219 on RASSF5A.[22] Furthermore, RASSF5A requires part of the switch region II of Ras in order to have maximal association with Ras in contrast to most Ras effectors that require only switch region I. Stieglitz et al. (2008) [22] have elegantly worked out the kinetic parameters of the Ras/RASSF5A association to show that Ras stays bound to RASSF5A for 10 seconds versus 60–250 milliseconds for Ras/PI3K or Ras/Raf associations. In addition, the Ras/RASSF5A RA complex has a surface area of contact at 1,546 Å versus 1,331 Å for Ras/Raf RBD and the Ras/RASSF1A complex had an association constant 33 times weaker than that of Ras/RASSF5A. These structural studies provide evidence for bonafide Ras association with some family members. Again, RASSF1A does not associate to classical Ras GTPases and any associations with Ras are indirect and most likely mediated through heterodimerization with RASSF5A, the only RASSF family member that can heterodimerize with RASSF1A.[16]

The RASSF apoptotic signaling pathways: keys to tumor suppressor function. As mentioned earlier, considerable excitement was generated when the RASSF family of proteins were first identified. RASSF5A demonstrated robust associations with Ras,[16] the ability to promote apoptosis in the presence of active Ras[23] and the ability to reduce the transforming ability of Ras in a mouse xenograft model.[24] Apoptotic function of RASSF2 and RASSF6 were also demonstrated to be augmented in the presence of active Ras, reinforcing the importance of the RASSF family in linking Ras signaling to apoptosis. RASSF1A, however, does not appear to strongly associate with Ras to promote apoptosis, suggesting that it no longer requires Ras and may utilize an as yet unidentified GTPase in order to promote apoptosis.

The majority of the pro-apoptotic function of RASSF5A is through the associations with mammalian sterile 20-like kinases, MST1 and MST2 (**Fig. 1**). MST is a cytosolic class II germinal center serine/threonine kinase that shows similarity to MAPKK and can be activated by both intrinsic and extrinsic stimuli. MST1 and MST2 share 76% sequence identity and contain an N-terminal catalytic domain followed by an autoinhibitory segment and a colied-coil Salvador/Rassf1a/Hippo (SARAH) domain that mediates hetero- and homo-dimerization.[25,26] Both MST1 and MST2 have been shown to undergo a caspase-3 (at $DEMD^{326}$ of MST1) and caspase 6/7 (at $TMTD^{349}$ of MST1) dependent cleavage following apoptotic stimulation.[27] This results in an enhanced kinase activity and ability to translocate

to the nucleus in order to phosphorylate histone H2B at serine-14. This phosphorylation event functions to possibly target H2B for endonuclease attack, promoting DNA fragmentation and subsequent cell death.[28] Regulating the activity of MST1/2 are a limited number of non-catalytic adapter proteins including RASSF family members that can associate through their SARAH domain.[27,29] Although important for associations with MST1/2, the SARAH domain of RASSF5A was demonstrated by Aoyama et al. (2004) [30] to be dispensable for its growth suppressive properties in A549 lung cancer cells. Therefore, it remains to be determined if MST1/2 are the only downstream effectors of the Ras/RASSF5A signaling pathway linked to the SARAH domain.

Similar to RASSF5A, RASSF1A can utilize its SARAH domain to associate with and activate both MST1 and MST2 in order to drive apoptosis and subsequent translocation of MST1 and MST2 to the nucleus.[20,29] It has been demonstrated that recombinant RASSF1A can inhibit the kinase activity of MST1 whereas overexpressed RASSF1A can activate MST1 in response to Fas/FasL stimulation[31] suggesting that the in vivo regulation of MST1 by RASSF1A involves more than the simple association of the two proteins. RASSF2 [16] and RASSF6 [32] can also form a complex with MST1/2 in order to activate the kinase activity of MST1/2. RASSF3 [27] was found to associate with MST in a yeast two hybrid screen suggesting that several RASSF family members may be physiological binding partners to MST1 and MST2. Curiously, the SARAH domain is absent from N-terminal RASSF family members suggesting that they may have lost the ability to associate with MST1/2 and instead most likely associate with a unique spectrum of interacting proteins. Furthermore, it is uncertain if MST1 and MST2 associate with RASSF family members while they are associated with the microtubular network. In summary, the RASSF/MST associations function to regulate MST catalytic activity in vivo and may direct MST to sites of activation and promote associations with endogenous substrates in order to induce apoptosis.

In addition to their roles in cell death, MST1 and MST2 have recently been shown to be involved in several aspects of biology ranging from regulation of organ size via Yes associated protein (YAP),[33] modulating hypertrophic responses in both cardiomyocytes and fibroblast cells of the heart[34] and regulation of actin cytoskeleton integrity by the activation of the JNK pathway.[35] Furthermore, MST1/2 can be activated by death receptor stimulation, IGF/Akt stimulation[36] and non-physiologically with okadiac acid.[27] Recently, the *Mst1*$^{-/-}$*Mst2*$^{-/-}$double knockout mouse was generated[37] that revealed that single loss of either gene was well tolerated and normal organ development was observed. However, the loss of both MST1 and MST2 resulted in the in utero death at approximately embryonic day 8.5. The *Mst1*$^{-/-}$*Mst2*$^{-/-}$ embryos exhibited severe growth retardation, failed placental development, impaired yolk sac/embryo vascular patterning and primitive hematopoiesis, increased apoptosis in placentas and embryos and disorganized proliferating cells in the embryo proper.[37] These findings indicate the essential roles for these two sterile 20-kinases in early mouse development, cell proliferation and survival.

We have also defined some of the molecular mechanisms of apoptotic regulation by RASSF1A that is MST-independent.[18,38,39] Ectopic expression of RASSF1A (and not RASSF5A) enhanced death receptor-evoked apoptosis stimulated by TNFα and TRAIL.[18] In contrast, knockdown (by RNA interference) or knockout cells to either RASSF1A or modulator of apoptosis-1 (MOAP-1, a downstream target to RASSF1A) have significantly reduced caspase activity, defective cytochrome *c* release, Bax translocation and impaired death receptor-dependent apoptosis.[38] Intrinsic pathway dependent cell death does not rely on death receptor stimulation but can promote decreased mitochondrial permeability, enhanced cytochrome *c* release and subsequent cell death. This pathway does not appear to be significantly lost in *Rassf1a*$^{-/-}$ cells and is reduced in *Moap-1*$^{-/-}$cells (unpublished observations). Our current model of RASSF1A mediated cell death in described in **Figure 3**. Death receptor stimulation primarily drives the activation of RASSF1A to complex with TNFR1/MOAP-1 at membrane proximal sites. This allows RASSF1A to expose the BH3-like domain of MOAP-1 to associate with and conformationally change Bax in order to drive Bax activation and localization to the mitochondrial membrane.[18,39] Bax can then proceed to promote cytochrome *c* release, activation, nuclear and cytoplasmic breakdown resulting in the demise of the cell.[38] Consistent with our model, the absence of MOAP-1 in H1299 non-small cell lung cancer cells resulted in the inability of RASSF1A to associate with TNF-R1 but association returned once MOAP-1 expression is re-established.[18] Importantly, robust RASSF1A/MOAP-1 association does not require the presence of active K-Ras contrary to what was observed by Vos et al. (2005).[40] We currently do not have an explanation for this but have observed RASSF1A/MOAP-1 associations in several cell types (including the ones utilized by Vos et al. [2005]) without the need to overexpress K-Ras or other GTPases. Reduced antibody efficiencies for the immunoprecipitations may explain these differences in addition to clonal differences in the cell type utilized. We believe that RASSF1A/MOAP-1 interactions are highly dynamic associations that are dependent on death receptor activation[38] and the ability of RASSF1A to promote apoptosis is dependent on the presence of MOAP-1.

Regulation of RASSF1A

The pro-apoptotic function of RASSF1A can be regulated on many levels. (1) Basal inactivation of RASSF1A is achieved by RASSF1A self association[18,39] and via 14-3-3 interactions (to isoforms σ and ε) at serine 175/178/179 of RASSF1A,[39] (2) TNFα stimulation functions to promote MOAP-1 recruitment to TNF-R1, disrupt RASSF1A self association and association with 14-3-3 and promote the recruitment of RASSF1A to TNF-R1/MOAP-1.[18,38] As previously mentioned, if MOAP-1 is absent RASSF1A does not associate with TNF-R1; and lastly, (3) the possible re-association with 14-3-3 may occur in order to prevent continued stimulation of this cell death pathway (unpublished results and see **Fig. 3**). The 14-3-3 family of proteins function as molecular scaffolds to restrict

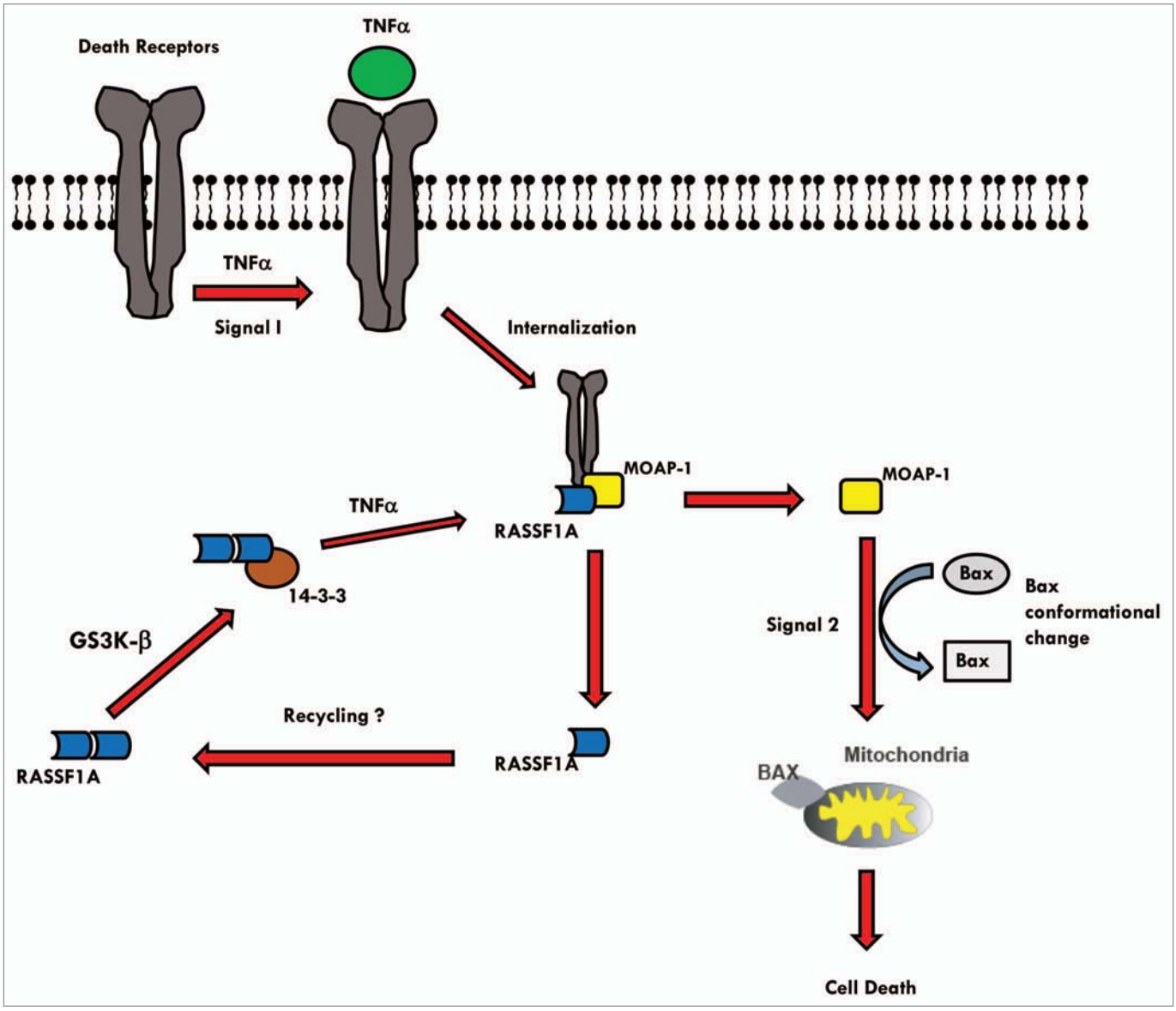

Figure 3. Model for RASSF1A modulated apoptosis. Death receptor-induced cell death (TNFα is used as an example) can result in the recruitment of protein complexes to activate Bax and promote apoptosis. Basally, RASSF1A is kept complexed with 14-3-3 by GSK-3β phosphorylation in order to prevent unwanted recruitment of RASSF1A to death receptor and uncontrolled stimulation of Bax and apoptosis. Once a death receptor stimuli has been received (TNFα as shown above), the TNFR1/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 re-associate with 14-3-3 to prevent continued stimulation of this cell death pathway (unpublished observations). Please see text for further details.

the localization, stability and/or molecular interactions of their target proteins such as Bad, Bax and apoptosis signal regulated kinase (ASK) 1.[41] In addition, both c-Raf and Raf-1/B-Raf can also be modulated by 14-3-3 associations.[42,43] 14-3-3 recognizes the phosphorylated serines at residues 175, 178 and 179 on RASSF1A. Mutation of these residues resulted in increased basal cell death in H1299 cells providing evidence that associations with 14-3-3 are important to provide basal inhibition of TNFα-dependent cell death linked to RASSF1A.[39] We further demonstrated that basal phosphorylation of RASSF1A is carried out by the canonical Wnt signaling pathway multifunctional serine/threonine kinase, GSK-3β. This adds an interesting dimension to the regulation of RASSF1A with links to the Wnt/β-catenin signaling pathways, an important pathway in neuronal development, embryogenesis and cancer.[44] What drives GSK-3β phosphorylation of serine 175 and possibly serine 178/179 of RASSF1A is an interesting question. In addition to GSK-3β, RASSF1A can also be regulated by phosphorylation by ATM,[19] PKC[45] and the cell cycle kinases (Aurora kinases).[46] These phosphorylations have been demonstrated to be important for the biology of RASSF1A with respect to cell cycle control and DNA damage response.[19]

Continued analyses of the role these phosphorylation events play in the biology of RASSF1A will yield a wealth of information of how the tumor suppressor properties of RASSF1A are regulated.

RASSF1A Polymorphisms: Key to Unknown Functions

Somatic mutations of RASSF1A are uncommon but several non-germline polymorphisms have been detected in tumors (mainly lung, breast, kidney and nasopharyngeal carcinomas) and in a few cell lines[47] that can be mapped to these functional domains (**Fig. 2**). The population distribution and significance of these alterations in tumorigenesis remains to be determined. Two 1A polymorphisms have been demonstrated to lose microtubule association (C65R and R257Q) [48,49] and two failed to induce cell cycle arrest by inhibiting cyclin D1 accumulation (A133S and S131F).[50] The ATM site polymorphisms, especially A133S is the most common RASSF1A polymorphism identified to date with two others also indentified to this region—an S131F and I135T change. Recently, it was demonstrated that S131 of the ATM site can be phosphorylated upon gamma radiation and phosphorylation of S131 is required to signal to p73, induce cell death and suppress colony formation.[19,50] The importance of these changes within the ATM phosphorylation site of RASSF1A is not currently known but we speculate that these changes may perturb the tumor suppressor properties of RASSF1A. As such, patients harboring the ATM site polymorphisms to RASSF1A may be resistant to radiation based chemotherapy. If this is true, one must not only determine if RASSF1A is present in cancer patients but if they have a polymorphic form of RASSF1A.

We have also characterized the importance of several polymorphisms in modulating the stability of tubulin and RASSF1A tumor suppressor function—C65R, E246K and A133S.[49] Interestingly, the C65R polymorphism acquires a nuclear localization and does not have the ability to promote cell death (unpublished observations). The majority of the other polymorphism to RASSF1A localize to microtubules in a similar manner to wild type RASSF1A (unpublished observations and ref. 35). In addition, we have preliminary evidence from a mouse xenograft assay to suggest that the SARAH domain polymorphisms (A33T and Y325C) and RA localized polymorphisms (V211A and R257Q) are transforming, suggesting that a functional SARAH and RA domains are required for the tumor suppressor function of RASSF1A (unpublished observations). The biological role of these alterations in tumorigenesis remains to be determined and a detailed analysis of the role of RASSF1A polymorphisms to the biology of RASSF1A is warranted.

Other Roles for RASSF Family of Proteins

Regulating cell death and cellular proliferation are common themes for the RASSF family of proteins. These functions have been well documented, interacting partners are being identified, and the importance of Ras binding for their function is becoming clearer. As we gain more knowledge of the biology of the RASSF family of proteins, we are beginning to realize that their role stretches beyond cell death and cell cycle regulation. RASSF1A has been demonstrated to be involved in the recovery from hypertrophic injury[51] and RAPL is an important component of lymphocyte adhesion and homing.[15] RASSF1A, RASSF5A, RASSF7 and RASSF10 have been shown to associate and localize to microtubules or to centromeres[13] while RASSF8 is an important part of adherens junction and may have a role in cellular migration.[14] Microtubule associations are very commonly observed amongst the RASSF family of proteins that may allow for the stability of tubulin, proper formation of the mitotic spindle and aid in sister chromatid separation during mitosis.[14] RASSF1A has also recently been shown to associate with C19ORF5/MA1PS complex and to localize to the microtubule-organizing center to anchor RASSF1A to the centrosomes.[52,53] Furthermore, C19ORF5/MA1PS can associate with LC3, an important component of the autophagosome and with the mitochondria-associated protein, leucine-rich PPR-motif containing protein (LRPPRC).[54,55] These associations may be important for autophagy involving proteins from both the microtubule and mitochondrial network. Autophagy or self digestion, is a major mechanism that occurs during starvation to reallocate nutrients from unnecessary biological processes to more urgent ones. It is a process that involves the formation of the autophagosome in order to rid the body of unwanted material through the lysosomal machinery. It may also be an important component of the tissue repair pathway allowing for efficient membrane fusion following injury. We have substantial evidence for the role of RASSF1A in regulating intestinal inflammation and possibly in the repair process following colonic epithelial cell damage (unpublished observations). We are currently exploring how RASSF1A may be involved in regulating inflammation and epithelial tissue repair and we speculate that the role of RASSF1A in intestinal epithelial repair may involve associations with the microtubule and autophagy related proteins. These are just some examples of the diverse roles of the RASSF family of proteins have in physiology. Several members of the RASSF family of proteins are frequent targets for promoter specific methylation and inactivation in cancer. Detailed analyses of the molecular mechanisms and identification of their molecular partners is warranted in not only cancer but other disease groups. Surprising discoveries are on the horizon for this gene family and we have only begun to understand their importance in biology.

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