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Ras signaling through RASSF proteins

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Abstract

There are six core RASSF family proteins that contain conserved Ras Association domains and may serve as Ras effectors. They lack intrinsic enzymatic activity and appear to function as scaffolding and localization molecules. While initially being associated with pro-apoptotic signaling pathways such as Bax and Hippo, it is now clear that they can also connect Ras to a surprisingly broad range of signaling pathways that control senescence, inflammation, autophagy, DNA repair, ubiquitination and protein acetylation. Moreover, they may be able to impact the activation status of pro-mitogenic Ras effector pathways, such as the Raf pathway. The frequent epigenetic inactivation of RASSF genes in human tumors disconnects Ras from pro-death signaling systems, enhancing Ras driven transformation and metastasis. The best characterized members are RASSF1A and RASSF5 (NORE1A).

Keywords

Ras; RASSF1A; NORE1A; apoptosis; signaling; p53

1. Introduction

Activated Ras stimulates a plethora of mitogenic signaling cascades that synergize to promote growth and transformation [1, 2]. So it should not be surprising that Ras is so frequently aberrantly activated in human tumors [3–5]. However, in addition to this powerful pro-growth/transformation activity, Ras also has the capacity to stimulate apoptosis and senescence [4, 6]. This apparent paradox may be explained as a feedback mechanism to prevent the prolonged survival of cell with excessive Ras activity. As the mitogenic

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pathways of Ras are mediated by multiple effectors, so are the cell death pathways. The main Ras death effectors identified to date are the RASSF family of proteins.

In 1998, Vavvas et al described the cloning of a gene coding for a Novel Ras Effector (NORE1A) [7]. The protein contained a consensus Ras Association (RA) domain and bound to activated Ras. The rat homolog (designated Maxp1) had already been identified in an unpublished screen for 2 hybrid binding partners of MSS4. MSS4 was thought to act as a Rab exchange factor, raising the possibility of a Ras/Rab connection [8]. In 2000, the related RASSF1 gene was identified serendipitously as a two-hybrid binding partner of the DNA repair protein XPA [9] and by homology searches for novel Ras binding proteins based on the sequence of Maxp1 [10]. Although containing an RA domain similar to that of NORE1A (RASSF5), some controversy arose as to whether RASSF1 proteins actually complexed with Ras [10] or did not [11]. The confusion may have arisen as RASSF1A appears to be K-Ras preferential and only binds the farnesylated form of the Ras protein [12]. Thus, experimental approaches using H-Ras or bacterially prepared Ras protein, which remains unfarnesylated, may have proved misleading. Subsequent investigations appear to have validated RASSF1A as a physiological K-Ras effector that can be precipitated in endogenous complex with activated K-Ras [13–15]. Further *in silico* homology searches resulted in the cloning of 4 other RASSF family members. These all interact with activated K-Ras in exogenous expression systems.

The RASSF family proteins all produce multiple isoforms via splicing or differential promoter usage [12, 16, 17]. Most of the smaller isoforms remain uninvestigated, but the main isoforms all contain RA domains, and usually act to inhibit cellular growth and survival. They all exhibit down-regulation by epigenetic inactivation, to some degree, in human tumors [12, 16, 18]. Lacking obvious enzymatic activity, RASSF proteins appear to function as scaffolding or sub-cellular targeting proteins. In addition to the six core RASSF family members, there are a further 4 related proteins designated NRASSF proteins due to their different structure [19]. The role of Ras in the regulation of these NRASSF proteins is insufficiently characterized to be considered here.

RASSF proteins have now been shown to link (or have the potential to link) Ras to pathways that modulate apoptosis, senescence, autophagy, inflammation and DNA repair. They can interact with multiple tumor suppressor proteins and link Ras to the precise control of the two most important tumor suppressors in human biology: p53 and Rb. Indeed, RASSF1A and NORE1A (RASSF5) may serve as tumor suppressor hubs. Consequently, it should not be surprising that Ras driven human lung tumors that also lose RASSF1A expression exhibit the worst prognosis [20]. Here we will describe the currently known role of RASSF proteins in Ras signaling. It seems highly likely there are many more to be identified.

2. RASSF1

Early work showed that K-Ras could use RASSF1 proteins as pro-apoptotic effectors [10]. The most commonly expressed isoforms from the RASSF1 gene are RASSF1A and RASSF1C. RASSF1A is epigenetically inactivated by aberrant promoter methylation at high frequency in a broad range of human tumors [12, 16, 18]. Moreover, up to 15% of tumors

may carry RASSF1A mutations that impair protein function [21, 22]. Thus, RASSF1A may be the most frequently inactivated tumor suppressor yet identified in human cancer. Considerable evidence supports the concept that the epigenetic inactivation of RASSF1A correlates with clinical disease [23]. Moreover, RASSF1A promoter methylation correlates with the worst prognosis in Ras driven tumors [20], and suppression of RASSF1A by shRNA promotes metastasis in mutant Ras cell lines [24]. In transgenic systems, RASSF1A loss enhances K-Ras induced proliferation [25].

The RASSF1C isoform does not appear to be frequently down-regulated in cancer by promoter methylation. It can demonstrate pro-apoptotic properties in experimental systems [10] and suppresses tumor growth *in vivo* [26]. However, it may also promote growth [27]. This may involve the activation of β -catenin [28] and src [29]. RASSF1C is less studied than RASSF1A and there is little information describing the role of RASSF1C in Ras signaling.

2.1. RASSF1A and Ras driven apoptosis

Two hybrid studies identified the MST kinases as direct binding partners of RASSF proteins [30]. As these kinases are pro-apoptotic [31], they seemed to be good candidates for the effector molecules of RASSF driven apoptosis. Indeed, it has been shown that RASSF1A is an essential component of MST kinase activation [32]. Subsequent work has shown that RASSF1A stabilizes the active, phosphorylated forms of the MST kinases by inhibiting dephosphorylation by PP2A [33]. MST kinases have a number of substrates that might explain their apoptotic effects, such as H2B and JNK [34, 35]. However, it is the role of MST kinases in the Hippo pathway that has garnered the most attention. In its simplest iteration, MST kinases (whose *Drosophila* homolog is called Hippo) phosphorylate and activate the LATs kinases which then phosphorylate the transcriptional regulators YAP and TAZ [36]. This event was shown to regulate the localization [37] of YAP/TAZ to restrict their oncogenic activity and ultimately the stability of YAP [38]. Further studies revealed that RASSF1A also acted to promote the formation of a complex between YAP and p73 in the nucleus, resulting in enhanced transcription of pro-apoptotic genes such as PUMA [39].

In seminal work from the Kolch group, activated K-Ras was shown to control the Hippo pathway via binding to RASSF1A and promoting MST activation [14]. Similar results were obtained in cardiac systems by Del Re et al [40]. However, somewhat surprisingly, later siRNA studies showed that YAP did not seem to be the most important target for Ras/RASSF1A/Hippo growth suppression. Instead, the key target seemed to be the master tumor suppressor p53 [14]. p53 impacts multiple biological pathways and serves as a transcription factor regulating growth and survival [41]. It is frequently mutated in cancer [42]. p53 is the subject of an intricate web of regulatory processes, but the most powerful is probably mediated by the MDM2 ubiquitin ligase [43]. MDM2 binds and ubiquitinates p53 to promote its degradation by the proteasome. RASSF1A can complex with MDM2 and promote its auto-ubiquitination leading to its proteosomal degradation [44]. In addition, MDM2 forms a complex with LATS2 [45] which serves to suppress the ubiquitin ligase activity of MDM2 in a kinase dependent manner. Both of these actions lead to enhanced p53 stability and may be induced by the K-Ras/RASSF1A interaction [14].

In addition to MST kinases, two-hybrid studies identified the protein MOAP-1 as a direct binding partner of RASSF1A [46, 47]. MOAP-1 is a regulator of the apoptotic executor Bax [48] and has tumor suppressor properties [49]. Activated Ras enhances the interaction between RASSF1A and MOAP-1 and this leads to Bax activation [46], translocation to mitochondria and apoptosis [47]. At least one tumor derived point mutant of RASSF1A is defective for the interaction with MOAP-1, suggesting that it may be important to repress tumor development.

It is possible that the Hippo signaling pathway and MOAP-1 synergize to activate Bax. Del Re et al showed that K-Ras uses RASSF1A to stimulate MST1 to phosphorylate Bcl-xL and promote its dissociation from Bax [15]. This is a Bax activating event. This whole process may involve positive feedback loops, as over-expression of MOAP-1 leads to upregulation of MST1 [49].

2.2. RASSF1A and the modulation of mitogenic Ras effectors

Although originally conceived of as a component of a linear signaling pathway from Ras to the apoptotic machinery, we now know that RASSF1A can also act to modulate the activity of mitogenic Ras effectors. The two best-characterized Ras mitogenic effector pathways are those of the Raf/MAPK pathway and those of the PI-3 kinase/AKT pathway [50]. MST2 is a RASSF1A binding partner, but a proteomic screen determined that MST2 can also bind to Raf-1 [51]. Raf-1 competes with RASSF1A for MST2 binding, thus suppressing MST2 activation and apoptosis [52]. However, MST2 binding serves to suppress Raf-1 activation, in part at least, by impeding the interaction of Raf-1 with active Ras [53]. Thus, RASSF1A levels may dictate the degree of Raf-1 activity. Counter-intuitively, this means that loss of RASSF1A expression can inhibit the Raf/MAPK pathway due to enhanced MST2 association with Raf-1.

MST2 does not appear to interact with B-Raf [51], however, MST1 does [54] and is inhibited by the process. We have found that RASSF1A appears to have little effect on MAPK signaling in mutant B-Raf driven cells but family member RASSF6 actually enhances the association of MST1 with B-Raf and suppresses the phosphorylation of ERK [55]. So B-Raf signaling may be under the influence of non-RASSF1A family members.

The second best characterized Ras mitogenic effector is PI-3 kinase which acts in large part via the anti-apoptotic kinase AKT [56]. RASSF1A was found to suppress the activating phosphorylation of AKT on residue 473 [57]. The exact mechanism for this effect remains obscure but this site is regulated by the TORC2 complex [58] and by the PHLPP family of phosphatases [59]. Moreover, cleaved MST kinases can inhibit AKT [60]. One of the substrates of AKT is actually Raf-1, where it phosphorylates residue 259, an inhibitory modification. RASSF1A inhibits AKT mediated Raf-1 S259 phosphorylation [57]. In a further layer of complexity, MST2 is also a substrate of AKT and the phosphorylation enhances MST2 association with Raf-1 kinase [61]. These complex relationships have been considered in detail in Nguyen et al [62].

The potential for further levels of Raf modulation by RASSF proteins also exists. It has recently been shown that RASSF1A can bind and inhibit src family kinases [29]. As src and

several family members positively regulate c-Raf by phosphorylation of tyrosine residue 341 [63], then RASSF1A may also modulate c-Raf by modulating its tyrosine phosphorylation status. Furthermore, Raf-1 can be regulated by PP1A-mediated dephosphorylation [64]. We have recently found that PP1A forms a direct complex with NORE1A which can regulate its activity [65]. Thus, RASSF proteins have an intimate and exquisitely complex role in regulating Ras signaling through Raf-1 and AKT (figure 1). The activation of the Raf/MAPK pathway should not be considered the simple linear pathway of the text books, but instead an integrated circuit with multiple feedback and control loops. Ras tumors which have lost RASSF1A expression may have a completely different Ras mitogenic output than Ras tumors that retain RASSF1A expression.

2.3. RASSF1A and the Rho pathway

Ras mediates some of its biological functions by activating the Rac/Rho proteins [66]. In part this is by using the Rho exchange factor TIAM1 as a direct effector [67]. Experimentally, inhibition of Rac/Rho represses Ras mediated transformation [68]. RASSF1A may impact Rho signaling at several points. First, it can directly bind to RhoA and suppress its transforming activity. This may occur via competition for other Rho effectors and by the modulation of Rho ubiquitination [69]. RASSF1A knockout has been reported to result in upregulation of Rac1 activity [70]. These observations suggest that RASSF1A may act to restrict any activation of Rac/Rho signaling by Ras. However, a recent report has shown that RASSF1A may activate the Rac/Rho exchange factor GEF-H1 by dephosphorylation to promote the activation of RhoB [24]. So the interplay of RASSF1A with Ras mediated signaling through Rac/Rho is likely to be complex.

2.4. RASSF1A and microtubule dynamics

Some of the earliest studies of RASSF1A described its ability to complex with and hyper-stabilize microtubules [71–73]. This ability is mediated by the direct binding of RASSF1A to multiple Microtubule Associating Proteins (MAPS) such as MAP1A, C19ORF5 and MAP4 [73–76]. It appears to be essential for the tumor suppressing function of RASSF1A [76, 77]. The stabilization is enhanced by the presence of activated K-Ras, and so RASSF1A connects Ras to the control of microtubule dynamics [72].

2.5. RASSF1A and β -Catenin

Estabaud et al demonstrated that RASSF1C bound the ubiquitin ligase SCF- β -TrCP and suppressed its ability to inactivate its classic mitogenic target β -catenin [28]. Downregulation of RASSF1A also led to stabilization of β -catenin, although RASSF1A did not appear to bind β -TrCP. Later studies were able to detect an interaction between RASSF1A and β -TrCP and showed that suppression of RASSF1A led to β -catenin stabilization [78]. The role of Ras in the modulation of the effects of RASSF1A on β -TrCP/ β -catenin remain uninvestigated. However, recent work has shown a close link between YAP/TAZ and β -catenin. YAP/TAZ been reported to be essential for β -TrCP recruitment to the destruction complex responsible for β -catenin protein regulation [79]. It is tempting to speculate that the K-Ras/RASSF1A interaction can impact both components.

2.6. RASSF1A and DNA repair/damage response

If DNA damage to a cell becomes too severe, an apoptotic DNA damage response is initiated to edit the defective cell out of the organism [80]. Thus, the DNA repair processes and apoptotic signaling are closely linked. The RASSF1A gene was first identified in a two-hybrid screen as an interaction partner of the DNA repair protein XPA [9]. It was not until many years later that the interaction between RASSF1A and XPA was confirmed to be physiological and an important role for RASSF1A in nucleotide excision repair identified [81]. RASSF1A has also been shown to play an important role in the stabilization of replication forks via BRCA2 [82]. The role of Ras in regulating the interaction of RASSF1A with XPA or BRCA2 is unclear. However, RASSF1A can be phosphorylated by the ATM/ATR kinases [83], which are important for maintaining replication fork integrity [84] and regulating Nucleotide Excision Repair [85]. Activation of Ras can promote ATR [86] and ATM activation [87]. ATR promotes the association of XPA with RASSF1A [81]. Moreover, a SNP point mutant of RASSF1A (A133S) with a defective ATM/ATR consensus phosphorylation site exhibited differential association with XPA [81] and a differential activation of the pro-apoptotic DNA damage response [39]. The ability of Ras to both bind RASSF1A and promote its phosphorylation by ATM/ATR may make teasing out the true role of Ras in the DNA repair/DNA damage response functions of RASSF1A quite complicated.

In addition to the regulation of apoptosis, microtubule dynamics, β -catenin, DNA repair and the DNA damage response, RASSF1A has also been implicated in modulating senescence [57], inflammation [88], protein acetylation [81] and multiple ubiquitin ligase systems [17]. The role of K-Ras in modulating these properties has not been characterized, but it seems reasonable to hypothesize that it has a significant part to play.

3. NORE1A (RASSF5)

Novel Ras effector 1 (NORE1), also termed RASSF5, was the first member of the RASSF family identified in a yeast two hybrid screen using activated Ras as bait [7] and is the closest homolog to RASSF1. Similar to RASSF1, there are multiple isoforms of NORE1, the two main isoforms being NORE1A and NORE1B, or RAPL [89, 90]. NORE1A appears to be ubiquitously expressed [91] whereas NORE1B function seems to be more specific to lymphocytic cells [92]. There is strong evidence that NORE1A is a tumor suppressor. Exogenous expression of NORE1A potently inhibits tumor cell growth [91] and inhibition of NORE1A expression enhances cell proliferation [93]. NORE1A^{-/-} MEFs are more readily transformed by Ras than wild type MEFs, which require p53 or Rb inactivation to permit Ras transformation [94]. Although not as extensively epigenetically silenced in primary tumors as RASSF1A [12], *NORE1A* is frequently inactivated in many human cancers by promoter methylation (Table 1) and loss of NORE1A is associated with a more malignant phenotype and enhanced Ras activity in primary tumors [13, 95]. In addition, inactivation of *NORE1A* through a translocation event is linked to the development of clear cell renal cell carcinomas, a rare form of familial cancer [96], confirming its role as a tumor suppressor *in vivo*. Importantly, NORE1A is a *bona fide* Ras effector in that it binds

activated Ras directly *in vitro* and *in vivo* and elicits its growth inhibitory functions in a Ras-dependent manner [7, 11, 30, 91, 97, 98].

3.1. NORE1A induces apoptosis

Like RASSF1A, NORE1A has no enzymatic activity and exerts its growth inhibitory functions by acting as a scaffold, regulating multiple pro-apoptotic pathways. As with RASSF1A, NORE1A binds to the MST kinases, linking Ras to the Hippo pathway [30]. However, unlike RASSF1A, NORE1A does not appear to activate the MST kinases [111] and the interaction between NORE1A and the MST kinases is not required for NORE1A-mediated growth inhibition [112], suggesting that NORE1A exhibits its growth suppressive effects independently of canonical Hippo signaling. Other apoptotic pathways regulated by NORE1A include death receptor-mediated apoptosis [94, 113]. NORE1A sensitizes cells to TNF- α - and TRAIL-mediated apoptosis, both *in vitro* and *in vivo* and this appears to be mediated by the stress-response MAP kinases [94]. TNF- α treatment results in the activation of both p38 and JNK kinases, but not ERK. However, in NORE1A-depleted cells, TNF- α -induced activation of p38 and JNK is severely reduced [94]. Similar results were found with a further member of the TNF-receptor superfamily, CD40. In bladder cancer cells, NORE1A is a crucial mediator of CD40L/CD40-induced cell death, which is dependent on JNK signaling and not ERK [113]. These data suggest that NORE1A regulation of death receptor-mediated apoptosis may be Ras-independent.

3.2. NORE1A is a powerful Ras senescence effector

While NORE1A clearly has functions in modulating apoptosis, its predominant function may be as a powerful senescence effector of Ras [65, 114]. Ras-induced senescence is a well established phenomenon that is a barrier which must be overcome in order for cells to become transformed and is crucial for the protection against the development of cancer [115–117]. The detection of Ras-mediated senescence in multiple systems, including cell culture systems, primary human tumors and animal models confirms it is a physiologically relevant process [115, 118–122]. Both the p53 and Rb pathways have been implicated in senescence [115, 123, 124], however the exact mechanisms by which Ras regulates these pathways is not well understood [125]. Recently it has been shown that NORE1A may link Ras to the regulation of both p53 and Rb activity.

Over-expression of NORE1A is as effective at inducing senescence as over-expressing activated Ras and loss of NORE1A impairs senescence and enhances Ras-mediated transformation [114]. The first evidence suggesting that NORE1A may be the link between Ras and p53-mediated senescence showed that NORE1A induces p53-dependent activation of p21^{CIP1}, a cyclin dependent kinase inhibitor associated with p53-dependent senescence [93]. Furthermore, inactivation of p53 and down-regulation of NORE1A expression are mutually exclusive in primary human tumors, suggesting they lie in the same pathway [93]. Subsequent studies confirmed that NORE1A is indeed a key factor in Ras-mediated senescence and provided further insight into the mechanism by which Ras mediates p53 activity [114]. Ras promotes the formation of a complex between NORE1A and HIPK2 [114], a kinase that can regulate the apoptotic/senescence balance of p53 by promoting discrete posttranslational modifications [126]. In addition, NORE1A scaffolds HIPK2 to p53

in a Ras-regulated manner [114], inducing acetylation of p53 at lysines 320 and 382, two posttranslational modifications associated with p53 senescence activity [127], while simultaneously reducing phosphorylation of serine 46, a pro-apoptotic modification of p53 [128, 129]. As a consequence, NORE1A expression results in the up-regulation of p53-regulated senescence markers such as p21^{CIP1} and down-regulation of p53 apoptotic mediators, such as Bax [114]. While loss of p53 severely impairs the ability of NORE1A to induce senescence, it does not completely abrogate it [114], suggesting the involvement of additional NORE1A-regulated senescence pathways. The other major pathway associated with Ras-induced senescence is regulated by the Rb tumor suppressor [115, 130], and downregulation of Rb suppresses NORE1A-mediated senescence [65]. Rb activity is regulated predominantly by a complex phosphorylation/dephosphorylation cycle involving inactivation by cyclin dependent kinases (CDKs) and activation by protein phosphatases [131, 132]. One of the major phosphatases that plays an important role in activating Rb is PP1A [133, 134]. Intriguingly, PP1A activity is regulated by Ras and contributes to Ras mediated senescence [135, 136]. PP1A was identified as a NORE1A binding partner in a yeast two hybrid screen [137]. Subsequent studies confirmed that this interaction is physiologically relevant and is regulated by Ras [65]. In fact, NORE1A scaffolds PP1A to Rb, enhancing its dephosphorylation and thereby activating it [65]. Thus, NORE1A links Rb to Ras-mediated senescence via PP1A.

NORE1A therefore appears to restrain the transforming properties of Ras by simultaneously activating both p53 and Rb, making it a powerful Ras senescence effector. Mechanistically, this involves scaffolding of a kinase and a phosphatase to p53 and Rb respectively, allowing Ras to qualitatively control the post-translational modifications of both p53 and Rb. Loss of NORE1A would thus subvert both the p53 and Rb senescence pathways, uncoupling Ras from p53 and Rb and allowing the pro-growth signals of Ras to predominate, thereby facilitating transformation. NORE1A expression is down-regulated in many human tumors (table 1) and correlates not only with the development of malignancy, but also with the loss of senescence markers [114], thus inactivation of NORE1A function may be a key factor in the ability of Ras-driven tumors to overcome the senescence barrier and illustrates why reduced NORE1A expression is frequently associated with Ras-driven tumors.

3.3. NORE1A modulates protein turnover

In addition to its functions in apoptosis and senescence, NORE1A may also serve to couple Ras to the control of protein turnover, thereby regulating additional growth promoting pathways such as Wnt/ β -catenin signaling. Wnt signaling is required during embryonic development for various differentiation events and is under tight control, mainly through regulation of β -catenin [138], the key downstream effector of the Wnt signaling pathway that acts as a transcriptional co-activator of TCF, activating Wnt responsive genes [139]. Dysregulation of β -catenin results in activation of Wnt signaling and is associated with many human tumors [138]. Recently, it has been reported that oncogenic Ras can activate the Wnt/ β -catenin signaling pathway [140]. NORE1A forms a Ras-regulated complex with β -TrCP, the substrate recognition subunit of the SCF β -TrCP E3 ubiquitin ligase [141, 142] and promotes the degradation of β -catenin resulting in the suppression of β -catenin signaling [141], linking Ras to the negative control of Wnt signaling. Therefore, loss of

NORE1A would uncouple Ras from the negative regulation of β -catenin activity, resulting in activation of Wnt signaling and the transduction of pro-growth signals, revealing a further mechanism by which NORE1A restrains the growth promoting activities of Ras.

The NORE1A/ β -TrCP interaction does not result in a general activation of the ubiquitin ligase activity of the complex to all targets, but is rather specific to a certain subset of targets [141]. NORE1A forms a complex with MDM2, the principal regulator of p53 [43, 143] and regulates the stability of MDM2 in a Ras-dependent manner [144]. The mechanism appears to involve poly-ubiquitination mediated by the NORE1A/ β -TrCP complex, in contrast to RASSF1A, which promotes MDM2 self-ubiquitination [44]. Thus NORE1A/Ras has multifaceted effects on p53, regulating its stability by destabilizing the major p53 antagonist (MDM2) and regulating its activity by modulating the posttranslational code of p53 [114].

The scaffolding function of NORE1A thus links Ras to multiple biological processes, all of which promote the growth inhibitory properties of Ras. Loss of NORE1A in human tumors dissociates Ras from these processes and allows the transforming properties of Ras to predominate.

4. RASSF2

Although not as extensively characterized as RASSF1A and NORE1A, it is now well established that RASSF2 has tumor suppressor properties. RASSF2 forms an endogenous, GTP dependent complex preferentially with K-Ras, as it appears to only interact with H-Ras weakly or not at all [13, 145, 146]. Over-expression of RASSF2 induces apoptosis and cell cycle arrest *in vitro* and inhibits tumor formation *in vivo* [146, 147]. Loss of RASSF2 expression promotes invasion and transformation [145, 148]. Like RASSF1A and NORE1A, RASSF2 expression is down-regulated in multiple human cancers by promoter methylation [79, 83–90]. Loss of RASSF2 expression dramatically enhances the transforming potential of K-Ras [145, 148] and there is a positive correlation between the inactivation of RASSF2 and K-Ras/BRAF mutations in primary tumors [148–150]. It can be argued that RASSF2 may be the most important member of the RASSF family as it is the only one that is essential for life [151].

4.1. Pro-apoptotic activity of RASSF2

The mechanisms by which RASSF2 imparts its pro-apoptotic functions likely differ from those of RASSF1A and NORE1A. RASSF2 is predominantly nuclear in localization [147, 152] and as with the other RASSF family members, RASSF2 lacks enzymatic activity and acts by binding to other pro-apoptotic effectors and modulating their activity [153–156], which can occur via Ras-dependent or independent mechanisms. One mechanism by which RASSF2 exerts its pro-apoptotic activity is by binding to and regulating the nuclear translocation of the PAR-4 tumor suppressor [155]. The interaction between RASSF2 and PAR-4 is K-Ras-regulated and K-Ras enhances the nuclear translocation of PAR-4 via RASSF2 [155]. Thus, RASSF2 links K-Ras to PAR-4-mediated apoptosis, and in tumors lacking RASSF2, this apoptotic pathway is subverted. RASSF2 also interacts with both the MST1/2 kinases [153, 154], core kinases of the Hippo signaling pathway [157], but it is unclear whether this interaction regulates Hippo signaling. The interaction between RASSF2

and MST2 stabilizes MST2 [153] whereas that with MST1 appears to stabilize RASSF2 [154]. In addition, while RASSF2 modulates the activity of the MST kinases towards various substrates that are not components of the Hippo signaling cascade, RASSF2 itself appears to be a substrate for the MST kinases [153, 154], suggesting that RASSF2 may be activated by the MST kinases and may regulate apoptosis independently of Hippo signaling. It is unclear at the moment whether the effects of RASSF2/MST signaling are Ras-regulated. RASSF2-mediated apoptosis may also involve activation of the JNK signaling pathway and inhibition of NF- κ B [154, 158], however the exact mechanism by which RASSF2 modulates these pathways or whether they are Ras-regulated, remains to be determined.

4.2. Diverse functions of RASSF2

The molecular mechanism(s) underlying RASSF2 function may be more complex. RASSF2 may also have functions in inflammation and immune cell chemotaxis [158] and a recent proteomics analysis using RASSF2 as bait identified several novel RASSF2 binding partners that function in diverse biological processes including epithelial-mesenchymal transition (EMT), regulation of redox homeostasis and protein post-translational modifications, the interaction with some of which is regulated by K-Ras [156]. Thus, while RASSF2 clearly functions as a pro-apoptotic mediator of K-Ras, it may also link Ras to the control of numerous biological processes that are critical for normal cell homeostasis, and loss of RASSF2 would result in de-regulation of these important processes. This may explain why RASSF2 is crucial for normal development [151].

5. Other RASSF family members

The remaining members of the RASSF family, RASSF3, RASSF4 and RASSF6, are not as well characterized as RASSFs 1, 2 and 5, yet the preliminary evidence available on the function of these proteins indicate that they all harbor tumor suppressor activity and may also act as Ras death effectors.

5.1. RASSF3

RASSF3 is the smallest member of the C-terminal RASSF family, is approximately 60% homologous to RASSF1 [90] and is ubiquitously expressed in human tissues [90]. In our hands, RASSF3 complexes with activated K-Ras in exogenous expression studies. Over-expression of RASSF3 inhibits cell proliferation and induces apoptosis [159–161], and loss of RASSF3 expression promotes growth, inhibits apoptosis, enhances cell motility and correlates with more invasive, metastatic tumors [159, 162]. In addition, RASSF3 expression is down-regulated in some human tumors by not only epigenetic silencing of the *RASSF3* promoter, but also gene deletion and other mechanisms which have yet to be elucidated [99, 102–105]. The mechanisms by which RASSF3 exerts its growth inhibitory effects are not well characterized, and are only beginning to be unraveled. RASSF3-mediated apoptosis is p53-dependent and Hippo pathway independent [161]. In fact, RASSF3 stabilizes p53 by interacting with MDM2 and promoting its degradation [161]. RASSF3 may also play a role in DNA repair as loss of RASSF3 results in defects in DNA repair and genomic instability [161]. Thus RASSF3 functions, in part, in a similar manner to NORE1A and RASSF1A by modulating p53 activity and DNA repair, respectively, however whether the exact

mechanisms involved are the same or unique, and whether these functions of RASSF3 are regulated by Ras, remains to be determined.

5.2. RASSF4

RASSF4 was the 5th member of the RASSF family identified and is more homologous to RASSF2 than to RASSF1A or NORE1A [163]. It binds to activated K-Ras via the effector domain, and promotes K-Ras-induced apoptosis [163]. Additionally, over-expression of RASSF4 inhibits tumor cell proliferation, colony formation in soft agar and invasion [163, 164]. Thus, it appears that RASSF4 has the properties of a Ras death effector. Like the other RASSF family members, RASSF4 expression is down-regulated in some human tumors [110, 163–165] and loss of RASSF4 expression has been linked to the maintenance of oral squamous cell carcinoma stem cells, where RASSF4 plays a role in inhibiting MAP kinase signaling [166]. However, somewhat contradictory to this, RASSF4 may also have the potential to be oncogenic. Expression of RASSF4 is elevated in some breast cancers and alveolar rhabdomyosarcoma [163, 167] and it binds to MST1 to inhibit the Hippo pathway, resulting in enhanced cell growth and bypass of senescence [167]. Thus RASSF4 may have diverse biological effects in different cell systems, and further work will be required to characterize the exact nature of RASSF4 function.

5.3. RASSF6

RASSF6 lacks the N-terminal extension domain of NORE1A and the microtubule association domain of RASSF1A, and shares the greatest homology with RASSF2 and RASSF4 [168]. It exhibits a distinct expression pattern compared to other RASSF family members and is epigenetically silenced in primary cancers, although to a lesser extent than RASSF1A and NORE1A [55, 110, 169]. RASSF6 binds directly to K-Ras via its RA domain and synergizes with activated K-Ras to induce apoptosis [168]. As with the other RASSF family members, RASSF6 exhibits tumor suppressor properties; over expression of RASSF6 inhibits cell proliferation and induces apoptosis whereas loss of RASSF6 promotes cell growth *in vitro* and tumor formation *in vivo* [55, 168, 170–173]. The mechanisms by which RASSF6 induces apoptosis are independent of the Hippo signaling pathway, as RASSF6 interacts with MST2 to inhibit Hippo signaling, an effect observed with the *Drosophila* RASSF protein [171, 174]. RASSF6 induces apoptosis by both caspase-dependent and independent mechanisms; similar to RASSF1A, RASSF6 forms a Ras-regulated complex with the Bax-activating pro-apoptotic protein MOAP-1 [168, 170, 171] and also induces caspase-independent apoptosis that is mediated by Apoptosis Inducing Factor (AIF) and Endonuclease G [170]. RASSF6 also induces apoptosis in a p53-dependent manner. As with RASSF1A, RASSF3 and NORE1A, RASSF6 forms a complex with MDM2, promoting its ubiquitination and subsequent degradation, thereby regulating the cell cycle and inducing apoptosis [172]. Additional apoptotic pathways regulated by RASSF6 may be mediated by JNK signaling [173]. RASSF2 also activates JNK signaling and induces apoptosis independently of the Hippo signaling pathway [154], therefore JNK signaling may be one potential mechanism by which Ras induces apoptosis through the RASSF proteins. RASSF6 down-regulation also results in genomic instability, suggesting a further role for RASSF6 in regulating DNA repair [172]. One further mechanism by which RASSF6 may function as a tumor suppressor is to inhibit NF- κ B activity [168, 175], a function that is

again shared with RASSF2 [158]. RASSF6 may keep Ras proliferative signals in check by inhibiting MAP kinase signaling [55], a similar effect observed with RASSF4 [166]. Thus RASSF6 has a number of potentially overlapping functions with RASSF2 and RASSF4, which may be due, in part, to the relatively high similarity between the two proteins.

7. Concluding remarks

Activation of Ras signaling is arguably the most common event in human cancer. Ras exerts its growth stimulatory effects by transducing mitogenic signals via its effector proteins. Ras can, paradoxically, also inhibit cell proliferation and under normal growth conditions, a delicate balance exists between the growth promoting and growth inhibitory signals of Ras. The ability of Ras to induce growth arrest is facilitated by the RASSF family of tumor suppressors, which couple Ras to a wide range of biological processes, including various pro-apoptotic and pro-senescent pathways (figure 2). Inactivation or loss of expression of these RASSF proteins, which occurs frequently in a wide range of human cancers, disconnects Ras from these growth inhibitory pathways and allows the growth promoting functions of Ras to predominate. However, the picture is complicated by the effects of RASSF proteins on Ras mitogenic signaling pathways. While the six members of the RASSF family share some overlapping functions, they also appear to have distinct modes of action. The effects of multiple family member loss in the same tumor have not been investigated. Ras driven, RASSF defective, tumors may exhibit a differential response to therapy. Whether this effect can be exploited to patient benefit remains to be seen.

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Fig. 1. RASSF1A modulates mitogenic Ras signaling

RASSF1A has a complex role in regulating Ras signaling through Raf-1 and AKT, primarily through its interactions with the MST2 and Src kinases and the PP1A phosphatase.

Activation of Raf/MAPK signaling therefore consists of multiple feedback/control loops and should be thought of more as a complex network rather than a linear pathway. The presence/absence of RASSF1A together with cell context will likely govern the output signals from Ras signaling through this pathway.

Fig. 2. Summary of the major biological processes modulated by RASSF1A and NORE1A
 RASSF1A and NORE1A interact with numerous partners to enable them to regulate diverse biological processes, including apoptosis, senescence, cell migration and protein stability. RASSF1A appears to be more apoptotic while NORE1A is a key Ras senescence effector. Although RASSF1A and NORE1A have unique functions, they may also co-ordinately regulate some of the same biological processes, such as modulating the stability of p53 by interacting with and regulating MDM2 and controlling Wnt signaling by modulating β -TrCP activity.

Table 1Primary tumors with methylated *NORE1A*

Tumor Type	Frequency	References
Pheochromocytomas	67%	[99]
Esophageal Squamous Cell Carcinoma	65%	[100]
Head/Neck Squamous Cell Carcinoma	58%	[101]
Colorectal Cancer	39%	[102]
Hepatocellular Carcinoma	35–38%	[13, 93]
Gastric Cardia Adenocarcinoma	36%	[103]
Clear Cell Renal Carcinoma	32%	[96]
Lung, NSCLC	24–34%	[89, 104, 105]
Pleural Mesothelioma	5%	[106]
Wilms Tumor	15%	[107]
Astrocytoma	4%	[108]
Neuroblastoma	3–50%	[109, 110]