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The N-terminal RASSF family; A new group of Ras association domain containing proteins, with emerging links to cancer formation.

Victoria Sherwood [*†], Asha Recino[*], Alex Jeffries[*], Andrew Ward[*], Andrew D Chalmers[*1].

[*], Centre for Regenerative Medicine, Department of Biology and Biochemistry, University of Bath, Bath, BA2 7AY, UK.

[†], Present address, Cell and Experimental Pathology, Lund University, Malmö University Hospital, S-205 02 MALMÖ, Sweden.

1, to whom correspondence should be addressed (e mail ac270@bath.ac.uk).

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Key words: RASSF7, RASSF8, RASSF9 and RASSF10, ubiquitin fold, tumour suppressor.

Abbreviations used: AP1, activator protein-1; C1, Protein kinase C conserved region; COSMIC, Catalogue of Somatic Mutation in Cancer database; C-terminal, carboxy terminal; DAAX, death-domain-associated protein; DAG, diacylglycerol/phorbol ester binding; ERM, Ezrin, Radixin, moesin; JNK, c-Jun-NH_2-kinase; NDR, nuclear Dbf2-related; N-terminal, Amino terminal; PAM, peptidylglycine alpha-amidating monooxygenase; P-CIP1, PAM C-terminal interactor 1; RA, RalGDS/AF6 Ras association; RB, Ras binding domain; RASSF, The Ras-association domain family.

Abstract

The Ras-association domain family (RASSF) has recently gained several new members and now contains ten proteins (RASSF1-10), several of which are potential tumour suppressors. The family can be split into two groups, the classical RASSF proteins (RASSF1-6) and the four recently added N-terminal RASSF proteins (RASSF7-10). The N-terminal RASSF proteins have a number of differences from the classical RASSF members and represent a newly defined set of potential Ras effectors. They have been linked to key biological processes, including cell death, proliferation, microtubule stability, promoter methylation, vesicle trafficking and response to hypoxia. Two members of the N-terminal RASSF family have also been highlighted as potential tumour suppressors. This review will summarise what is known about the N-terminal RASSF proteins, addressing their function and possible links to cancer formation. It will also compare the N-terminal RASSF proteins to the classical RASSF proteins and ask whether the N-terminal RASSF proteins should be considered as genuine members, or imposters in the RASSF family.

Introduction

Ras proto-oncogenes form part of a superfamily of small GTPases comprising of five families; *Ras*, *Rho*, *Rab*, *Ran* and *Arf* [1]. They play a pivotal role in a myriad of cellular processes, including cell growth, apoptosis, adhesion, migration and differentiation [2, 3]. Unsurprisingly then, defects in Ras signalling can result in disease progression, in particular oncogenesis. Indeed, Ras mutations resulting in signalling aberrations, frequently occur in human tumours, particularly in pancreatic and lung adenocarcinomas (Catalogue of Somatic Mutation in Cancer database (Cosmic) [http://www.sanger.ac.uk/genetics/CGP/cosmic/]). Ras proteins carry out their diverse functions by binding to a broad range of Ras effectors and blocking these interactions has been highlighted as an important therapeutic opportunity that could be exploited for cancer treatments [4]. However this requires a better understanding of the effector pathways utilised by Ras [4].

Each Ras effector contains one of a number of Ras binding domains, as example is the RalGDS/AF6 Ras association (RA) domain. This conserved domain is the defining feature of members of the Ras association domain family (RASSF). The family now contains 10 members (RASSF1-10) which are split into two groups, the classical (RASSF1-6) and the amino terminal (N-terminal) RASSF proteins (RASSF7-10) [5]. Members of the classical RASSF proteins have been implicated in a range of biological processes, including the regulation of cell death, cell cycle control and microtubule stability, and are generally regarded as tumour suppressors. This has prompted great interest in these proteins and there are excellent reviews which mainly focus on the classical RASSF family [6-8] and in particular, RASSF1A [9-11]. Recently, four other proteins have been added to the family [5] and renamed RASSF7-10 (Table 1). These N-terminal RASSF proteins represent a new group of potential Ras effectors which may have important biological functions, some of which could well be distinct from previously studied Ras effectors. They may also have a role in cancer progression. In this review we will focus on the N-terminal RASSF proteins. We will summarise what is known about this newly described group of

proteins and ask is there any evidence to suggest a role for these proteins in cancer formation? We will also address the question of whether they should be considered as long lost members or imposters in the RASSF family.

RASSF proteins are defined by the presence of a Ras association domain/ ubiquitin fold

The defining feature of the RASSF proteins is the presence of a RA domain. This domain was identified by comparing sequences from different Ras binding proteins [12] and is present in over 50 human proteins (SMART database: http://smart.embl-heidelberg.de/). However, the RA nomenclature is potentially misleading as it implies that a protein with this domain will bind Ras. In fact, the binding affinities of RA domains for members of the Ras family show a huge variation and not all will bind Ras [13, 14]. A good example of a RA domain which does not bind Ras is found in the class IX myosin protein, myr 5 [15]. All RA domains are believed to form a similar three dimensional structure called a ubiquitin fold [16], however the RA domain in myr 5 lacks positively charged amino acids which are required for Ras binding [15]. It is not surprising that only a subset of RA domains bind Ras, as the sequences of different RA domains are highly divergent [12]. There are also other ubiquitin fold containing proteins, such as FERM domain containing proteins and ubiquitin, which do not interact with Ras [16]. Another possible cause of confusion is the fact that other Ras effectors such as Raf and PI3K interact with Ras through a domain called a Ras binding (RB) domain. Despite the difference in nomenclature this domain also forms a ubiquitin fold [16]. Thus, Raf, PI3K, RASSF proteins, FERM domain proteins and ubiquitin all share a common structural domain and can be considered part of a ubiquitin fold family [13]. The variation in ability to bind Ras means that a key step in studying RA/ubiquitin fold proteins, such as the RASSF family members, is to establish if the proteins function as Ras effectors, something which will be discussed below.

The classical and N-terminal RASSF proteins have different domain architectures

The RA domain/ubiquitin fold of classical RASSF members is found near the carboxy (C)-terminal of the protein, adjacent to a protein-protein interaction domain called the SARAH domain (Fig. 1). This domain is named after the 3 types of proteins that contain it; Salvador (WW45 in vertebrates), RASSF and Hippo (MST1/2 in vertebrates) [17]. SARAH domains have two α-helices which form a novel dimeric anti-parallel helix [18]. Dimerisation between SARAH domains allows Salvador, RASSF and Hippo to form homo and heterodimers. RASSF1 and 5 also contain a diacylglycerol/phorbol ester binding (DAG) domain (Fig. 1), known as protein kinase C conserved region (C1). In RASSF5/Nore1 the C1 domain can form an intramolecular complex with the RA domain/ubiquitin fold and when free bind the lipid phosphatidylinositol 3-phosphate[19].

The N-terminal RASSF proteins have a different domain architecture to the classical RASSFs (Fig. 1A). The RA domain/ubiquitin fold of the N-terminal members is located at the opposite end to the C-terminal location found in the classical RASSF proteins. The RA domains/ubiquitin folds of the two groups also have quite different sequences which form phylogenetically distinct groups (Fig. 2). In addition to the differences in RA domains/ubiquitin folds the N-terminal RASSF members lack an identifiable SARAH motif [5, 17]. However, some caution may be required on this

point. The SMART database predicts that RASSF7, 8 and 10 have extensive regions of coiled coil and like SARAH domains, coiled coils can form dimers mediated by hydrophobic residues [20]. Structural studies are required to confirm there is no similarity between the coiled coils of the N-terminal RASSF proteins and SARAH domains of the classical proteins.

RASSF7, 8 and 10 are all located close to members of the *Ras* family in the genome [5, 21, 22]. This suggests that the N-terminal RASSF proteins may have co-evolved with members of the *Ras* family. We have not found a similar association between the classical *RASSFs* and members of the *Ras* family, so this unusual juxtaposition of a *Ras* gene and a potential Ras effector represents another distinction between the two groups. The separation between N-terminal and C-terminal RASSF genes is not a recent event, *Drosophila* and *C elegans* have both classical (*dmRASSF* [23] and *T24F1*.3 [24]) and N-terminal RASSF (Table 1 and [5]) homologues. The differences between classical and N-terminal RASSF proteins prompted us to suggest they are distinct families, with the N-terminal RASSF proteins representing a new group of RA domain/ubiquitin fold containing proteins [5].

Classical RASSF proteins act as tumour suppressors

The focus of this review is the N-terminal RASSF proteins, however, before covering these proteins in detail we will summarise what is known about the six classical RASSF (RASSF1-6) proteins. This is not intended to replace comprehensive reviews of the family [6-8], but to allow a comparison with the N-terminal RASSF members.

RASSF1 was originally identified in a yeast-2-hybrid screen and the gene was found to reside at chromosome 3P21.3 [25], a region long suspected to contain at least one tumour suppressor [26]. The expression of one of the *RASSF1* transcripts, *RASSF1A*, was found to be repressed by promoter hypermethylation in lung tumours [25]. Subsequent studies found that *RASSF1A* was inactivated by methylation in a wide range of tumours (e.g. [27]) and it quickly emerged that *RASSF1A* is one of the most frequently methylated genes in cancer [9-11]. Restoring expression of *RASSF1A* reduces tumour growth and knocking out *RASSF1A* in mice causes an increased frequency of tumour formation [28, 29]. These studies provide convincing evidence that *RASSF1A* is a tumour suppressor, which is inactivated in a wide range of cancers. Inactivation by promoter methylation occurs with several other members of the classical RASSF family in human tumour cells, including *RASSF2, 4 and 5* [6-8], suggesting that they may also be tumour suppressors. Understanding why RASSF1A and other members of the classical RASSF family act as tumour suppressors has been far from straight forward, due to the variety of biological roles they possess. Classical RASSF proteins have been linked to a range of processes, particularly the regulation of apoptosis, cell cycle progression and microtubule stability.

Classical RASSF proteins are key regulators of apoptosis

RASSF family members have been linked to promoting apoptosis through a number of effectors [6-8]. One group of effectors are the proapoptotic kinases MST1 and MST2, which bind members of the classical RASSF family [24, 30, 31]. Hippo, the Drosophila homologue of MST1, forms part of an important tumour suppressor network which is crucial for growth control [32, 33]. Hippo functions by regulating the kinase Warts which in turn regulates the transcriptional activator Yorkie, which controls apoptosis-associated genes. Recent work shows that RASSF1A induced

apoptosis acts via a similar pathway, which involves MST2 activating LATS, causing the release of YAP which promotes transcription of p73 [34]. NDR (Nuclear Dbf2-related) kinases, which are related to LATS kinases, can also function down stream of MST1 to promote apoptosis [35]. In addition to MST1/2, classical RASSF proteins bind another positive regulator of apoptosis, MOAP1 [36-38]. After death receptor signalling, MOAP-1 and RASSF1A are recruited to the death receptor where the interaction of RASSF1A with MOAP-1 allows MOAP-1 to activate Bax and promote apoptosis [36, 39].

Classical RASSF proteins are important for microtubule stability and cell cycle progression

A second function of the classical RASSF proteins is to regulate the cell cycle. Expression of RASSF1A blocks cell cycle progression at a number of stages, including G_1, G_2-M and in prometaphase [40-43]. The RASSF1A induced arrest in G_1 is associated with reduced activity of c-Jun-NH_2-kinase (JNK) [44] and activator protein -1 (AP1) [45], which both promote cell cycle progression. RASSF1A also up regulates expression of the cyclin dependant kinase inhibitor $p21^{Cip1/Waf1}$[46]. These effects are likely to be mediated by a number of effectors. RASSF1A can bind MDM2 and death-domain-associated protein (DAAX). This prevents degradation of the tumour suppressor p53, which would allow p53 to promote cell cycle arrest [47]. RASSF1A can also bind and increase the activity of p120 E4F, a transcriptional repressor of Cyclin A2 [48, 49].

The RASSF1A induced arrest in mitosis is tightly associated with the ability of RASSF1A to associate with microtubules. RASSF1A can bind and stabilise microtubules [41, 43, 50, 51], probably via interacting with a number of microtubule associated proteins [50, 52]. Once bound to microtubule associated proteins RASSF1A appears to function as a scaffold, recruiting multiple regulators of mitosis. Current data suggests these may include Mst1/2, Cdc20, Aurora-A and Ran. Mst1 could signal through the Hippo pathway (see above) to regulate mitotic progression [53]. RASSF1A can also bind and inhibit Cdc20, which activates the anaphase promoting complex [54], although it should be noted that this interaction is controversial [55]. RASSF1A is phosphorylated by the mitotic kinase, Aurora-A [56], and can regulate the activity of Aurora-A [57]. Finally it has recently been shown that Ran can act as a RASSF1A effector to regulate microtubule stability [58]. In addition to mitosis RASSF1A has been linked to cell migration, which is consistent with a role in regulating microtubules [59].

Other members of the classical RASSF family have been linked to cell cycle progression. An example is RASSF5/Nore1, which shows striking similarities to RASSF1A. RASSF5/Nore1 can associate with microtubules [60] and suppress growth by a mechanism which involves p53 activating the expression of $p21^{Cip1}$ [61]. In summary, classical RASSF proteins have been linked to apoptosis, cell cycle control and the regulation of microtubule stability, all of which may contribute to the tumour suppressor function of these proteins.

Classical RASSF proteins and Ras

The presence of a RA domain/ubiquitin fold suggests the classical RASSF proteins will act as Ras effectors. However, as discussed above, not all RA domains bind Ras, and for many of the classical RASSF proteins it is not clear if they function as Ras

effectors to mediate the processes described above. RASSF5/Nore1 is perhaps the best documented Ras effector of the RASSF family. The splice variant RASSF5A/Nore1A was identified as a Ras interacting protein by yeast-two-hybrid and the endogenous protein interacts with Ras following addition of EGF [62]. RASSF5A/Nore1A is also the first member of the RASSF family to have the crystal structure of its RA domain/ubiquitin fold determined [63]. This was carried out in complex with Ras and demonstrated that the region which interacts with Ras is extended compared with other Ras effectors. This lengthened interface provides the RASSF5A/Nore1A – Ras complex with a prolonged lifetime compared to other Ras effectors. However, a physiological role for a RASSF5A/Nore1A-Ras complex is yet to be identified. The splice variant RASSF5B/Nore1B (also known as RAPL) is a Ras effector with a well documented physiological role in T cell signalling, where it associates with the Ras protein Rap1 [64].

RASSF1A can bind Ras in a GTP dependant manner [65], but it binds with a much lower affinity than RASSF5/Nore1 [63, 66]. This raises the question, is RASSF1A a genuine Ras effector? An endogenous RASSF1A-Ras complex has been described [67], but similar to the RASSF5A/Nore1A-Ras complex, the physiological role of this complex is not known. A recent twist to this story is that RASSF1A can bind to the small GTPase Ran [58] and regulate microtubule organisation (discussed above). The binding appears direct and can be seen with endogenous protein. Ran is not a member of the Ras family but is part of the large superfamily of related small GTPases [1]. This suggests that RASSF1A functions by binding Ran in addition to, or instead of, binding Ras. It also raises the possibility that RASSF1A and other RASSF proteins might bind other small GTPases in addition to Ras and Ran. Future work is required to untangle the biology of the classical RASSF proteins and the role that Ras and other small GTPases play in their function.

The N-terminal RASSF family

The difference in domain architecture and sequence of the RA domains prompted us to propose that the N-terminal RASSF proteins are a distinct family from the classical RASSF proteins [5]. This makes the decision to add them to the RASSF family look questionable. However, one crucial benefit of the renaming is to group the N-terminal RASSF proteins together for the first time. This makes it possible to compare what is known about each member. To achieve this we have searched the literature for work relating to each N-terminal RASSF protein. We used the 11 different names which have been given to the vertebrate members (Table 1) and it is important to point out that many of the references cited here use the older nomenclature. Given the importance of the work on the Drosophila classical RASSF protein [23], we have also looked at the three potential N-terminal Drosophila members. In the following sections we will summarise what is known about each of the N-terminal RASSF proteins and where appropriate relate it back to what is known about the classical RASSF proteins.

RASSF7: The first RASSF protein to be described?

RASSF7 was originally identified by a study which set out to sequence genes which are located close to *HRas* in the genome [21]. The authors found an unstudied gene and called it *Hras1 cluster 1*, *HRC-1*. *HRC-1* was recently renamed *RASSF7*, presumably because the protein it encodes contains an RA domain/ubiquitin fold and was not part of a recognised family. The hypothesis of the authors who identified

RASSF7/HRC-1 was that it might be a growth regulator because it was close to *HRas* in the genome [21]. They also suggested, based on Southern blotting, that RASSF7/HRC-1 might be part of a large family of related proteins. This was six years before RASSF5/Nore1 was identified as a potential Ras effector [62] and it is only recently that the authors predictions about RASSF7 have begun to be confirmed.

The genomic position of *RASSF7* places it in close proximity to the *HRAS1* minisatellite which is immediately down stream of *HRas*. Rare alleles of this minisatellite were shown to be associated with cancer risk [68, 69] and it was proposed that altered expression of *RASSF7* might contribute to the increased risk [70]. This generated a great deal of interest in the region, however subsequent studies using improved technology failed to find a link [71, 72] and the idea that rare alleles of the minisatellite are associated with cancer risk has fallen from favour.

***RASSF7*: a hypoxic response gene which is up regulated in certain cancers.**

The advent of genomic screening technology has made it possible to interrogate the entire genome for genes which are misregulated in cancer. Several microarray studies have shown that *RASSF7* is up regulated in cancer (Table 2). One example is in pancreatic cancer. Two independent studies have found that *RASSF7* expression is increased in pancreatic ductal adenocarcinoma relative to normal tissue [73-75]. In addition to ductal adenocarcinoma, *RASSF7* has increased expression in a second type of pancreatic cancer, islet cell tumours [76]. This study selected *RASSF7* as a key gene whose expression can be used to identify islet cell tumours. *RASSF7* expression is also increased in endometrial cancer [77]. Similar to the situation in islet cell tumours, *RASSF7* showed a large increase in expression and was selected as one of the top 50 genes which distinguish malignant from normal endometrium. Finally *RASSF7* lies in a genomic region which is amplified in ovarian clear cell carcinoma and its expression is increased in these cancers, correlating with the genomic amplification [78].

Interestingly, recent work offers plausible explanations as to why *RASSF7* may be up regulated in cancer samples. Hypoxia, which occurs in solid tumours, is known to cause a large number of gene expression changes [79] and *RASSF7* expression was found to be up regulated by hypoxia in MCF7 breast cancer cells [80] and in human umbilical vein endothelial cells [81]. This predicts that the hypoxic environment found in solid tumours would cause an increase in *RASSF7* expression. Furthermore, *RASSF7* is also down-regulated by the tumour suppressor, BRCA1, suggesting its expression would be increased in cancer cells which have lost BRCA1 function [82].

RASSF7 is required for cell death and proliferation

An important question is what role RASSF7 plays in these cancerous cells? There is currently no evidence to suggest that increased expression of RASSF7 promotes cancer formation. However, RASSF7 function has been linked to some key biological processes including the regulation of cell death and proliferation. RASSF7 has been shown to be required for necroptosis [83], a regulated form of necrosis which is distinct from apoptosis. A large scale siRNA screen was carried out to find proteins required for necroptosis and this identified RASSF7 and RASSF8.

We identified *Xenopus RASSF7* in a microarray screen [84] and subsequently found that in *Xenopus*, RASSF7 is essential for cell cycle progression and cell survival [5].

In cells where RASSF7 is knocked-down, mitotic spindles fail to form and cells arrest in mitosis. This causes nuclear fragmentation and apoptosis. Consistent with a role in mitotic progression, *Xenopus* RASSF7 is localised at the centrosome. However, *Xenopus* RASSF7 is not a core component of the centrosome, rather it appears to be enriched at the centrosome because it interacts with the minus ends of microtubules. Preliminary data from a large scale screen suggests that the *Drosophila* homologue may also be required for cell proliferation. Knockdown by RNA interference caused a reduction in the mitotic index and weak spindle defects [85].

RASSF7, like other RASSF proteins, contains no catalytic domain so to understand its function it is crucial to identify the proteins it interacts with. Yeast-2-hybrid studies have identified potential binding partners for human RASSF7. These include CHMP1B, which is associated with endosomal membrane trafficking, and DISC1 which interestingly interacts with microtubules [86, 87].

RASSF8 is located in a genomic region associated with lung cancer risk

The sequence for *RASSF8* was first deposited into the NCBI data base by Hoon and Yuzuki in 1996 (accession number Q8NHQ8) and called '*human carcinoma associated HoJ-1*'. However, there appears to have been no publication associated with this submission. Subsequently, *RASSF8* was characterised as a gene involved in a chromosomal translocation associated with a complex type of synpolydactyly [88, 89]. *RASSF8* was found to be located on chromosome 12 and referred to as *C12orf2*. The chromosomal translocation fused *RASSF8* with the *fibulin-1* gene (22q13.3). It is believed that this disrupts a *fibulin-1* splice variant, causing the synpolydactyly. C12orf2 was then renamed RASSF8, presumably because it contains a RA domain/ubiquitin fold and was not part of a recognised family. This occurred at the same time as HRC-1 was renamed RASSF7 and both proteins were added to the RASSF family.

RASSF8 is located about 700 Kb from the *KRas2* gene [22] and both genes lie in a region called Pals1. This region has been identified as a major susceptibility locus in a mouse model for lung carcinogenesis [90]. There are a number of genes in this region but it is mutations in the *KRas2* gene that are believed to be responsible for the increased risk [91]. The homologous region in humans has also been associated with increased lung adenocarcinoma risk [92]. However, in humans it is not clear if it is *KRas2* that is responsible. Analysis of the region in a Japanese population, identified the D12S1034 microsatellite as being most tightly associated with lung cancer risk [93]. The D12S1034 locus showed a bigger difference between cases and controls than the microsatellite adjacent to *KRas2*. This argues that in some human cancers, susceptibility may be due to a mutation in a gene adjacent to D12S1034 rather than in *KRas2* itself. *RASSF8* lies within 20 Kb of D12S1034 making it a good candidate gene, particularly as RASSF8 has also been described as a potential tumour suppressor in lung cancer (see below). However, common polymorphisms in *RASSF8* are not associated with cancer risk in an Italian population [94]. Thus, currently it is not clear if there is the link between *RASSF8* and the increased lung cancer risk associated with the Pals1 region.

RASSF8 is a potential tumour suppressor

There are several lines of evidence to suggest that RASSF8 may be a tumour suppressor (altered expression levels are summarised in Table 2). The best

characterised example is in lung cancer [22]. In lung adenocarcinoma *RASSF8* transcript levels were reduced compared with normal tissue. Over-expression of RASSF8 protein in lung cancer cell lines also inhibited anchorage-independent growth, which has been correlated with tumour progression and metastasis. In addition to lung adenocarcinoma, *RASSF8* expression is also down-regulated in male germ cell tumours [95], despite the fact that the gene lies in a genomic region which shows gain in almost 100% of these cancers. Finally, *RASSF8* was identified as a candidate gene involved in leukaemia and lymphoma formation in a study on retroviral-induced blood cancers in mice [96]. This model assumes that oncogenes and tumour suppressors often lie near common retroviral insertion sites. A genomic region next to *RASSF8* was targeted seven times, making it one of the most frequently hit sites in the study. This suggests that mis-regulation of *RASSF8* may contribute to leukaemia and lymphoma formation, so it is interesting that *RASSF8* has higher expression in human hematopoietic stem cells and is required for blood cell development in Zebrafish [97]. It is not known why RASSF8 might be a tumour suppressor but it is interesting that, similar to RASSF7, it is required for cell death by necroptosis [83]. This form of cell death may be particularly important in cells with deficiencies in their apoptotic machinery, such as tumour cells, so a role in necroptosis would be consistent with a tumour suppressor function.

Mass spectrometry and yeast-2-hybrid screens have identified a number of potential binding partners for RASSF8; including the scaffolding protein, 14-3-3γ which binds phosphoproteins to modulate their function [98]; and FRMD4, an ERM (Ezrin, Radixin, Moesin) protein that links membrane domains to actin and PSMD4, a component of the proteosome [99]. These potential binding partners offer interesting leads for future work aimed at understanding why RASSF8 may function as a tumour suppressor.

RASSF9 is a Ras binding protein that has been linked to vesicle trafficking

RASSF9 was first identified by a yeast-2-hybrid screen as a protein that interacted with the cytoplasmic domain of peptidylglycine alpha-amidating monooxygenase (PAM) [100]. Based on this interaction it was named PAM C-terminal interactor 1 (P-CIP1). PAM is a transmembrane protein found in secretory vesicles of neurons and endocrine cells, where it catalyses the α-amidation of bioactive peptides such as oxytocin and vasopressin. This modification is essential for the activity of these peptides [101]. We realised that PCIP-1 contains an RA domain/ubiquitin fold and is closely related to RASSF7+8 and suggested it should be renamed RASSF9 [5]. The binding of RASSF9/P-CIP1 to PAM was confirmed and RASSF9/P-CIP1 was found to associate with recycling endosomes [102]. This led to the model that it might bind the cytoplasmic domain of PAM during recycling of the enzyme [102], an interaction that may be regulated by phosphorylation as the cytoplasmic domain of PAM is known to be multiply phosphorylated [103]. However, *RASSF9* mRNA is expressed much more widely than *PAM*, so RASSF9 might have additional roles, perhaps binding other transmembrane proteins.

Interestingly RASSF9 is the one member of the N-terminal RASSF proteins which has been shown to bind RAS proteins. Pull down experiments with RASSF9 and Ras family GTPases showed that RASSF9 binds N-Ras, K-Ras and R-Ras [14]. An issue that remains to be addressed is whether RASSF9 binds endogenous Ras, or other

small GTPases, something which has not been straightforward to answer for the classical RASSF proteins (see above).

RASSF10 is a candidate tumour suppressor in childhood leukaemia

We discovered that a predicted protein was similar in sequence to RASSF9 and named this protein RASSF10 [5]. This gene was completely unstudied, until recent work showing that it is a candidate tumour suppressor in childhood leukaemias [104]. The transcript of *RASSF10* was characterised and found to be shorter than the predicted version. The protein encoded by the shorter version is more similar to RASSF7-9 and is used in Figure 1. *RASSF10* contains a large CpG island and given the frequent inactivation of classical RASSFs by promoter hyper methylation (see above) the authors examined the methylation status of this gene in childhood leukaemia. They found that *RASSF10* was frequently methylated in leukaemia cell lines (100%) and T cell acute lymphocytic leukaemias (T-All) (88%), but not in normal bone marrow and blood samples. *RASSF10* was also rarely methylated in B-ALL (16%). Inhibiting this methylation caused an upregulation of expression in the leukaemia cell lines. This data strongly suggests that *RASSF10* expression is inhibited by promoter methylation in a high percentage of T-All, raising the possibility that RASSF10 might function as a tumour suppressor in these cancers. ESTs for *RASSF10* are present in a number of tissues [104] and it will be interesting to see if *RASSF10* is methylated in tumours derived from these tissues.

The function of RASSF10 remains unstudied in vertebrates. There is a potential *Drosophila* homologue (Table 1). However little is known about this gene except that it is expressed in precursors of the peripheral nervous system [105], and knocking down its function impairs Hedgehog signalling [106]. RASSF10 offers exciting opportunities for future study.

Concluding remarks

The N-terminal RASSF proteins have a different domain architecture from the classical RASSF proteins and so we proposed that they should be considered as a separate family [5]. Donninger and colleagues came to a similar conclusion for RASSF7 and 8, suggesting that they are a separate sub-family distinct from the ‘true’ RASSF proteins [10]. If the N-terminal and classical RASSF proteins are members of different families then one might expect that there will be little overlap between their biology. However, RASSF7 and RASSF1A show similar centrosomal localisation and mitotic defects when knocked down and RASSF10 and members of the classical RASSF family both show promoter hypermethylation. These similarities might suggest that the N-terminal RASSF proteins are genuine RASSF proteins. However, we feel the differences between them still outweigh the similarities and that the N-terminal RASSF proteins are not ‘true’ RASSF proteins, but a separate family. Emerging evidence presented in this review suggests that the N-terminal RASSF proteins might play a role in tumour formation (summarised in Fig. 3). There is now an exciting opportunity to study this new group of proteins in more detail and confirm whether they are important in oncogenic progression.

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Figure Legends

Figure 1. N-terminal RASSF proteins are structurally distinct from the classical RASSF proteins.
The N-terminal RASSF proteins comprise a recently identified set of RA domain/ubiquitin fold containing proteins. Their domain architecture is distinct from the classical RASSF proteins suggesting they should be considered as a separate group. Sequences used for the domain analysis were; hsRASSF1A (NP_009113), hsRASSF2 (NP_055552), hsRASSF3 (NP_835463), hsRASSF4 (NP_114412), hsRASSF5/splice variant NORE1A (NP_872604), hsRASSF6B (NP_958834), hsRASSF7 (NP_003466), hsRASSF8 (NP_009142), P-CIP1/RASSF9 (AAD03250) and RASSF10 (NP_001073990, the short version described in [104]). Adapted from Figure 1 in [5].

Figure 2. The RA domains of the classical and N-terminal RASSF proteins are phylogenetically distinct.
The phylogeny of the RA domains from RASSF1-10, ten other RA domains from nine other proteins (AF6 has two RA domains), and a yeast outlier was inferred. The analysis was carried out by profile aligning the RA domains to the alignment of RA domains from the SMART database, using ClustalW. Phylogenetic inference was then carried out using neighbor-joining, parsimony and maximum likelihood methods from PHYLIP3.67. There was some variation in the tree topologies, but with each method the RA domains from the classical and N-terminal proteins clustered in two well separated monophyletic groups. The tree here shows the maximum likelihood inference using the Jones-Taylor-Thornton model. RA domains from the following sequences were used; RASSF1A (NP_009113.3), RASSF2 (NP_055552.1), RASSF3 (NP_835463.1), RASSF4 (NP_114412.2), RASSF5 (NP_872604.1), RASSF6 (NP_803876.1), RASSF7 (NP_003466.1), RASSF8 (NP_009142.2), RASSF9 (AAD03250.1), RASSF10 (NP_001073990.1), STE50 (P25344), Rap1 (AF478469_1), A4beta (EAW86096.1), GRB10 (Q13322), MYOIXb (Q13459), GRB7 (NP_005301.2), DGKtheta (P52824), PhosC (Q5VWL4), AF-6 (BAA32485.1).

Figure 3. Emerging evidence suggests a possible link between the N-Terminal RASSF proteins and cancer progression.
A summary of the evidence suggesting that the N-terminal RASSFs may play a role in tumourigenesis. Data consistent with potential anti-tumourigenic roles are indicated by red arrows, whilst evidence suggesting pro-tumourigenic roles are indicated by green arrows. Broken arrows highlight links which may be anti- or pro-tumourigenic. The cellular localisation is given where it is known. Full details and references are provided in the review text.

Tables

N-terminal RASSF member	Chromosome	Alternative names	Potential *Drosophila* homologue	Potential *C. elegans* homologue
RASSF7	11p15.5	HRAS1 cluster1 (HRC1), C11orf13	CG5053	K05B2.2*
RASSF8	12p12.3	Human carcinoma associated HoJ-1(HoJ-1), C12orf2		
RASSF9	12q21.31	peptidylglycine alpha-amidating monooxygenase (PAM) C-terminal interactor 1 (P-CIP1), PAMC1	CG13875*	
RASSF10	11p15.2	similar to peptidylglycine alpha-amidating monooxygenase C-terminal interactor 1	CG32150*	

Table 1. N-terminal RASSF family.
The table summarises the nomenclature used for the N-terminal RASSF proteins. The genes marked with a * are not predicted to have an RA domain by the SMART database, however they do have sequence similarity over the RA domain region of the vertebrate N-terminal RASSF protein, thus can be considered as potential homologues.

Gene	Tumour type	Change	Reference
RASSF7	Pancreatic adenocarcinoma	Up-regulated	[74] + [75]
	Islet cell tumour	Up-regulated	[76]
	Endometrial carcinoma	Up-regulated	[77]
	Ovarian clear cell carcinoma	Amplified and up-regulated	[78]
RASSF8	Lung adenocarcinoma	Down-regulated	[22]
	Male germ cell tumours	Down-regulated	[95]
RASSF10	T cell acute lymphocytic leukaemia	Down-regulated by promoter methylation	[104]

Table 2. Aberrant expression of the N-terminal RASSFs in cancer cells.
The table presents a summary of manuscripts reporting aberrant expression of N-terminal RASSF members in cancer. There are other examples in the Oncomine data base (http://www.oncomine.org/), but we have only included those where we can find reference to the N-terminal member in the primary paper or supplementary material. It is important to note that these papers often use the alternative gene names described in Table 1.

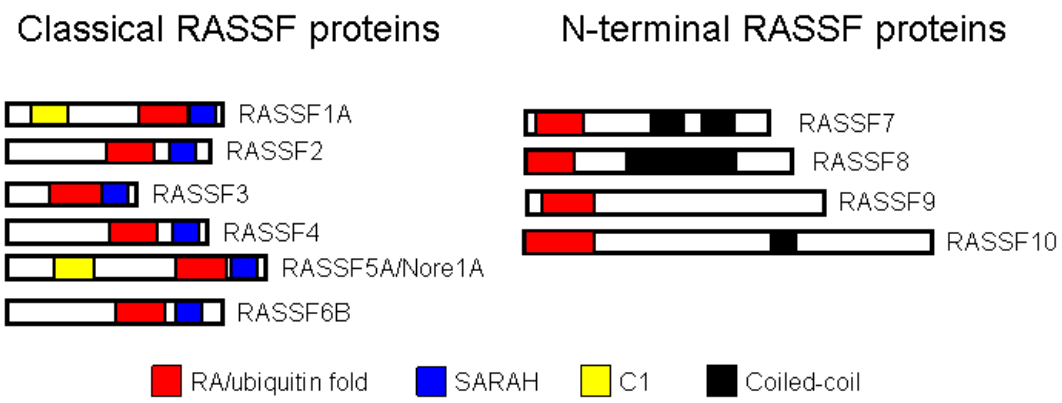

Figure 1. Sherwood et al.

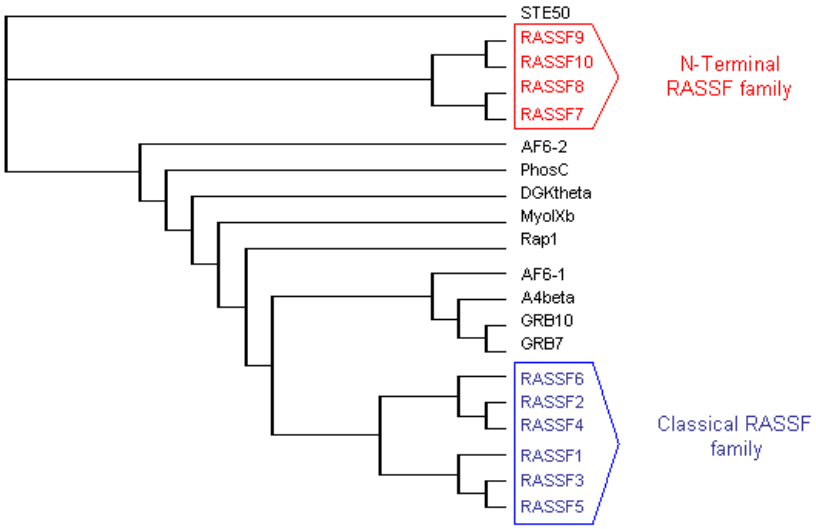

Figure 2. Sherwood et al.

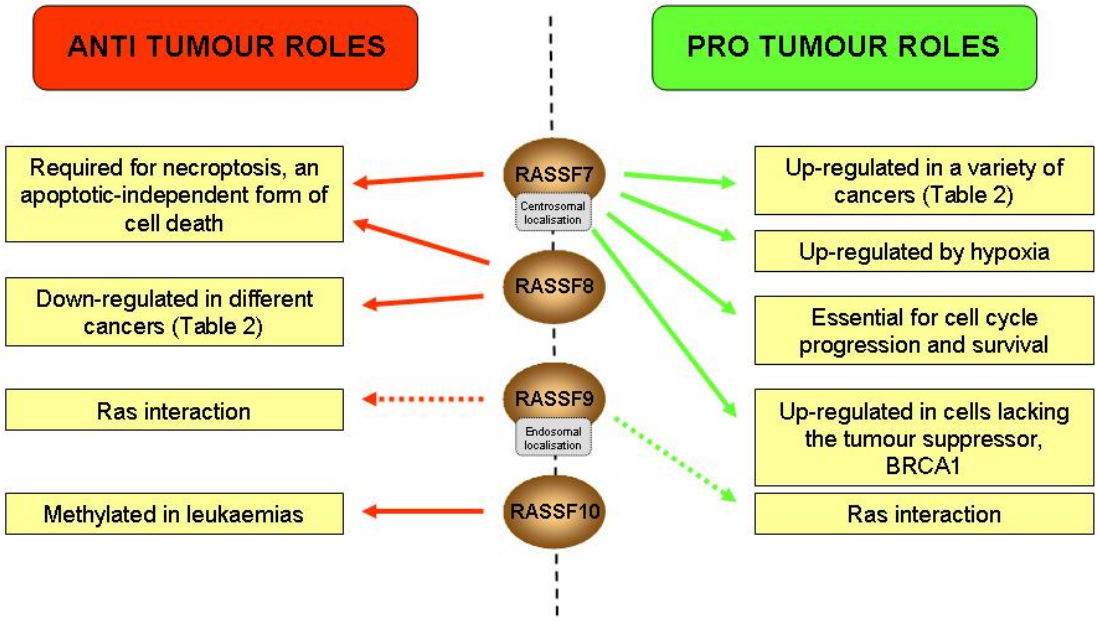

Figure 3 Sherwood et al.