Biochimica et Biophysica Acta xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbacan

Review

The RASSF proteins in cancer; from epigenetic silencing to functional characterization

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ARTICLE INFO

Article history:
Received 28 November 2008
Received in revised form 19 March 2009
Accepted 21 March 2009
Available online xxxx

Keywords:
RASSF
Tumor suppressor
Microtubule
RAS
Apoptosis
Epigenetics

ABSTRACT

The Ras-Association Domain Family (RASSF) comprises ten members, termed RASSF1 to RASSF10. RASSF1 to RASSF6 harbor a C-terminal Ras-association (RA) domain and RASSF7 to RASSF10 contain an N-terminal RA domain. Interestingly, it was observed that in various tumor types distinct RASSFs transcripts (e.g. *RASSF1A* and *RASSF2A*) are missing due to hypermethylation of their CpG island promoter. Since methylation of the *RASSF1A* promoter is described as an early and frequent event in tumorigenesis, *RASSF1A* could serve as a useful diagnostic marker in cancer screens. RASSFs are implicated in various cellular mechanisms including apoptosis, cell cycle control and microtubule stabilization, though little is known about the underlying mechanisms. Tumor suppressing functions were reported for several members. Here we review the current literature on RASSF members focusing on structural, functional and epigenetic aspects. Characterizing the cellular mechanisms that regulate the signaling pathways RASSFs are involved in, could lead to a deeper understanding of tumor development and, furthermore, to new strategies in cancer treatment.

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Abbreviations: AP-1, Activator protein-1; APC/C-CDC20, complex anaphase-promoting complex/cyclosom-cell division cycle 20 homolog; ATM, ataxia telangiectasia mutated; C19ORF5, chromosome 19 open reading frames 5; CDC20, cell division cycle protein 20; CDH1, Cdc20 homolog 1; CDK, cyclin dependent kinase; CNK1, connector enhancer of KSR; DAXX, death-domain associated protein; DNMTs, DNA-methyltransferases; Hpo, Hippo; Id-1, Inhibitor of DNA binding/differential-1; LATS, large tumor suppressor; MOAP-1/MAP1, modulator of apoptosis-1; MAP1B, microtubule-associated protein 1B; MST, mammalian sterile 20-like kinase; MTOC, microtubule organizing center; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NORE1, Novel Ras Effector 1; PKC, protein kinase C; RA, Ras-association (RalGDS/AF-6) domain; SARAH, Salvador, RASSF and Hippo; Sav, Salvador; SKP2, S-phase kinase-associated protein 2; TNFα, tumor necrosis factor alpha; Wts, Warts; WW45, human Salvador homolog; YAP1, Yes-associated protein 1

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doi:10.1016/j.bbcan.2009.03.004

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1. Introduction

This review will give an insight into the broad spectrum of investigations regarding the Ras-Association Domain Family (RASSF), which has been studied with ever-growing interest since the first description and characterization of RASSF1 in the year 2000 [1].

Ras-Association Domain Family 1 (RASSF1) was first identified using a (presumably false-positive) yeast-two-hybrid screen through its interaction with XPA, a protein known to function in DNA excision repair [1]. The C-terminus of RASSF1 displayed a high homology to mouse Nore1 (later termed Rassf5), a known Ras effector [2]. Dammann et al. further identified three different RASSF1 transcripts: *RASSF1A*, *B* and *C*. These variants were shown to share four common exons, encoding a Ras-association *(RalGDS/AF-6)* domain, from which the name of the family was derived. The Ras-Association Domain Family 1 (*RASSF1*) gene is located on the small arm of chromosome 3 (3p21.3) [1]. Earlier loss of heterozygosity (LOH) studies were investigating the very same chromosomal region in various tumor entities and had already proposed the existence of tumor suppressor genes at 3p21.3 [3,4].

Different cellular mechanisms can contribute to tumorigenesis. Loss of function of tumor suppressor genes and gain of function of proto-oncogenes can be observed [5]. Molecular changes lead to the activation of oncogenes by mutations, amplifications, increased promoter activity and/or translation of fusion-proteins. Regarding tumor suppressor genes, loss of one allele can be compensated by the intact remaining one, therefore, the crucial inactivation is the silencing of the second allele [6]. Loss of function of tumor suppressors can occur through mutations, deletions, mitotic recombination events or by epigenetic inactivation, through methylation of CpG islands in the promoter region [7]. The term Epigenetics comprises all hereditary changes of gene regulation that are not based on the DNA sequence itself [8]. So-called CpG islands are DNA regions in which dinucleotides of cytosine–guanine are statistically overrepresented in comparison to the whole genome. CpG islands are often associated with promoters; genes, whose promoters are especially rich in CpG sequences, tend to be expressed in most tissues. Cytosines in CpG islands can be methylated at the 5-position by DNA-methyltransferases (DNMTs) and the corresponding gene (e.g. tumor suppressor gene) can, therefore, be silenced. Methylated CpGs are recognized by methyl-CpG-binding proteins that can form histone deacetylase silencing complexes that regulate gene expression at the chromatin level [9–11]. Epigenetic inactivation of the tumor suppressor RASSF1A was frequently reported in different tumor entities as reviewed previously [12,13].

2. The Ras-Association Domain Family

The Ras-Association Domain Family comprises ten members from RASSF1 to RASSF10 as well as various isoforms, which are listed in Fig. 1. One characteristic features of this family is the Ras-association domain (RA), which can be found either C-terminally (RASSF1 to RASSF6) or N-terminally (RASSF7 to RASSF10). The other characteristic feature is the Sav–RASSF–Hpo (SARAH) domain, encoding a protein–protein interaction domain, which however is only found in RASSF1 to 6. Prominent and most intensely studied family members are RASSF1A (an isoform of RASSF1) and RASSF5, also called NORE1. Whereas RASSF7 to RASSF10 joined the family only recently and therefore little data exist to date.

3. The RASSF domains

The Ras-Association Domain Family proteins contain several distinct domains that are depicted for each member in Fig. 1. The **RA domain** is a Ras-association (RalGDS/AF-6) domain and a characteristic feature of Ras-effectors and Ras-related-GTPases [14], that gave its name to the whole Ras-Association Domain Family [1]. The **C1 domain** was named after its high homology with a cysteine-rich diacylglycerol/phorbol ester (DAG)-binding domain also called protein kinase C conserved region 1 (C1). Its central C1 zinc finger [15] is characteristic for the domain and RASSF1A was shown to associate with the TNF-R1/MOAP-1 or TRAIL-R1/MOAP-1 complex via its C1 domain [16]. The **ATM domain** corresponds, by its sequence, to a putative ATM-kinase phosphorylation motive. It was shown, that this peptide becomes phosphorylated by ATM at least *in vitro* [17]. The ATM kinase (ataxia telangiectasia mutated) is of central importance for the regulation of cell cycle checkpoints that lead to DNA-repair and apoptosis [18]. However, a functional relevance of this ATM domain has not been confirmed *in vivo* yet. The **SARAH domain** is a protein–protein interaction domain, named after the tumor suppressors *Sal*vador (in *D. melanogaster*; orthologue of human WW45), *RA*SSF and *H*ippo (in *D. melanogaster*; orthologue of the human proapoptotic kinase MST1). This domain is characterized by its length of 50 amino acids and its distal C-terminal position. SARAH domains are found in WW45, MST1 and different RASSFs and heteromeric and as well as homomeric interactions can be conducted via SARAH domains [19], e.g. MST and WW45 or RASSF1A and RASSF5 interaction [20–22]. Ortiz-Vega et al. reported that recombinant RASSF1C exhibits a much weaker ability to homodimerize or heterodimerize with RASSF5 in comparison to RASSF1A [20]. Regarding RASSF2 it was shown to associate with RASSF3 and RASSF5 [23] and RASSF5 with RASSF1A through their nonhomologous amino-terminal segments [20]. The functional consequence of these RASSF interactions need to be addressed in further studies, as it is known that SARAH domains play a central role in the newly discovered Hippo signaling pathway in *D. melanogaster*, which regulates cell proliferation and apoptosis [19,24].

4. The RASSF members

4.1. RASSF1

The *RASSF1* gene, which is located on the small arm of chromosome 3 (locus 3p21.3) codes for eight exons (1α, 1β, 2αβ, 2γ, 3, 4, 5 and 6) (Fig. 2). There are seven different RASSF1 isoforms (RASSF1A to RASSF1G) that are generated by differential usage of two promoters (distance 3.5 kb) and through alternative splicing [25]. So far however, the biological relevance of only two isoforms, RASSF1A and RASSF1C, was demonstrated. Regarding the transcripts *RASSF1B* and *RASSF1E* there is currently not enough evidence to support a biological role, as well as for the candidates *RASSF1F* and *RASSF1G* that possibly enter

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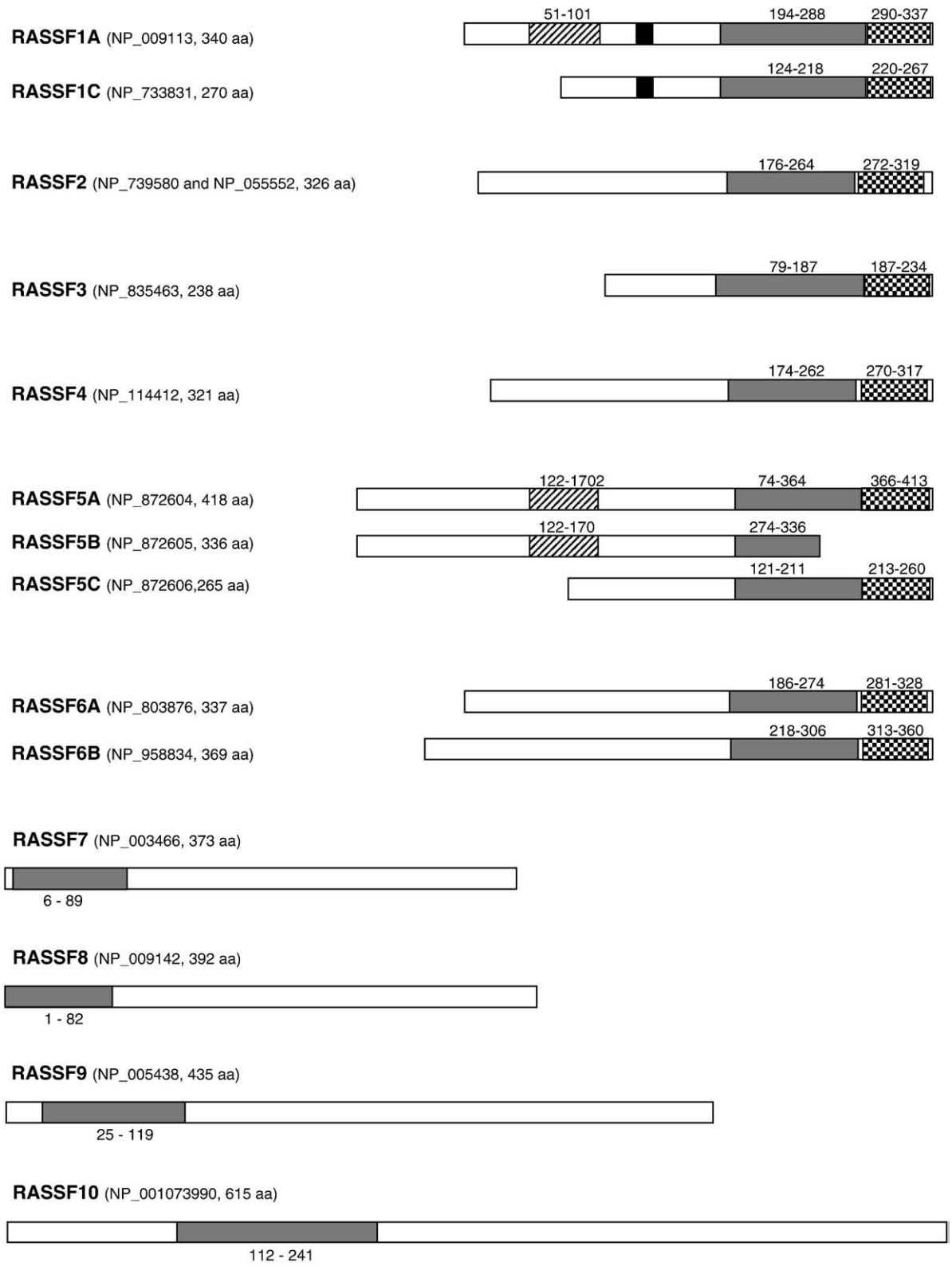

Fig. 1. The Ras-Association Domain Family (RASSF). Family members are listed according to Entrez Gene (http://www.ncbi.nlm.nih.gov/sites/entrez) with accession number and length in amino acids (aa). Only major isoforms are depicted and conserved protein domains were determined with Prosite (http://www.expasy.ch/prosite/). Characteristic domains are the protein kinase C conserved region (C1; striped), the putative ATM-kinase phosphorylation site (black), Ras-association (RalGDS/AF-6) domain (RA; grey) and the Sav–RASSF–Hpo interaction site (SARAH; checkered).

the nonsense-mediated mRNA decay. The two main variants are RASSF1A and RASSF1C containing a RA domain, SARAH domain and ATM domain, whereas the C1 domain can only be found in RASSF1A. The isoform A is being transcribed from the upstream promoter and isoform C from the downstream promoter (Fig. 2) and both promoters are located within CpG-islands. However only the upstream promoter

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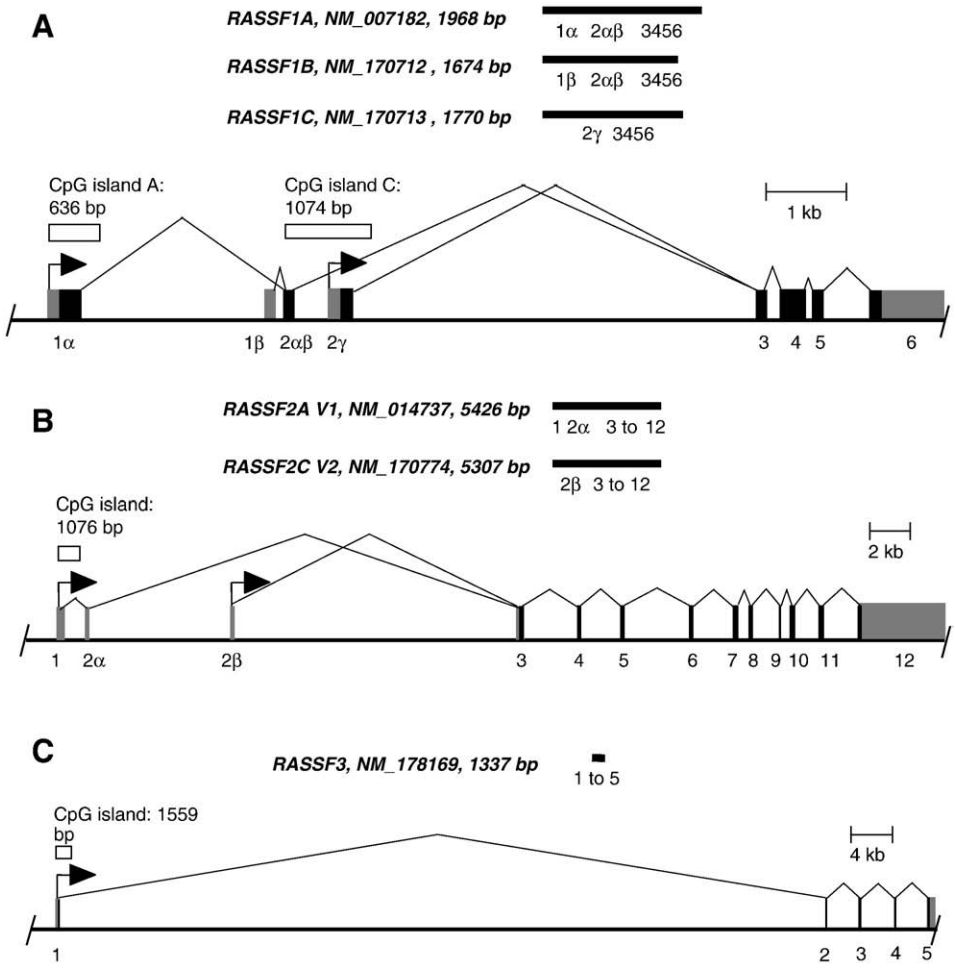

Fig. 2. Genomic arrangement of *RASSF1* at 3p21.3, *RASSF2* at 20p12.1 and *RASSF3* at 12q14.2. Organization and transcripts are depicted according to Entrez Gene (www.ncbi.nlm.nih.gov/sites/entrez) with accession numbers and length in bp. Black and grey boxes represent coding and untranslated sequences, respectively. Transcription start sites are marked by arrows. Location and size of CpG islands at promoter regions were determined by CpG plot (www.ebi.ac.uk/emboss/cpgplot/). (A) *RASSF1* codes for eight exons termed 1α , 1β , 2αβ , 2γ, 3, 4, 5 and 6. The transcripts are spliced into three isoforms *RASSF1A*, *RASSF1B* and *RASSF1C*. *RASSF1A* and *RASSF1C* are transcribed from two distinct CpG island promoters. (B) *RASSF2* codes for thirteen exons termed 1, 2α, 2β and 3 to 12. The two transcripts, RASSF2A (Variant 1; V1) and *RASSF2C* (Variant 2, V2) are shown. Only RASSF2A is transcribed from a CpG island promoter. (C) RASSF3 codes for five exons. RASSF3 is transcribed from a CpG island promoter.

is often hypermethylated in various tumor entities [1,13]. Both isoforms are ubiquitously expressed in non-tumor tissues [1], but in tumors and tumor cell lines, the transcript of *RASSF1A* is frequently missing due to CpG island methylation of its promoter [12,13]. The search for mutations in different tumor types regarding the *RASSF1A*-exons resulted in only few alterations, mostly polymorphisms [25]. It has been shown that a polymorphism at codon 133 in RASSF1A is associated with tumor alterations and an early onset of breast cancer in BRCA1/2 mutation carriers [26,27].

The reexpression of RASSF1A in cancer cell lines led to a reduced proliferation rate [1,28–31] and furthermore, it was shown that Rassf1a-knockout-mice are prone to develop cancer in an advanced age [32–34]. Hypermethylation of the *RASSF1A* promoter correlated in certain tumor types with a poor prognosis for the patients and an advanced tumor stage [35–38]. Interestingly, *RASSF1A* promoter methylation was also implicated in metastasis. Liu et al. showed that metastasis from neuroendocrine tumors into lymph nodes is related to *RASSF1A* promoter methylation [39]. Zhang et al., who investigated small bowel carcinoids, found the *RASSF1A* promoter more frequently methylated in metastatic compared to primary tumors [40]. Taken together, these and other studies show *RASSF1A* to be a prominent and epigenetically silenced tumor suppressor.

Agathanggelou et al. and Chow et al. demonstrated that over-expression of RASSF1A in nasopharyngeal carcinoma, a non-small cell lung cancer cell line and neuroblastoma cell lines leads to a set of differentially expressed genes [41,42]. These studies could help to further reveal the underlying mechanisms of RASSF1A tumor suppression.

For RASSF1C, the other major RASSF1 isoform, there are less consistent studies. Although no hypermethylation of the promoter was detected [1,28], its transcript was missing in some cancer cell lines [43], and this could be attributed to deletions of 3p21.3, which are frequently observed in cancer [28]. In one report, it was stated that the inactivation of RASSF1C during *in vivo* tumor growth identified it as a tumor suppressor gene [44]. In contrast in another study, it was reported that RASSF1C stimulates human lung cancer cell proliferation [45].

4.2. *RASSF2*

The *RASSF2* gene, which is located on chromosome 20 (locus 20p12.1), is being transcribed into two different isoforms (Fig. 2). Isoform A and C encode the same protein containing the SARAH domain as well as the RA domain (Fig. 1; online: NCBI, Entrez Gene, 2008), that shows 28% identity to that of RASSF1A and 31% identity to that of RASSF5. RASSF2 however lacks the cysteine-rich domain of RASSF1A and RASSF5 [46]. *RASSF2A*'s first noncoding exon is located within two CpG islands (Fig. 2) [23]. The first exon (2β) of *RASSF2C* is only 20 bp long and so far only one GenBank entry supporting its presence exists (online: NCBI, Entrez Gene, 2008). The *RASSF2A*

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transcript can be detected in most normal tissues [46], but down-regulation of *RASSF2A* by promoter hypermethylation has been shown in different tumor cell lines and primary tumors [23,46–50]. Akino et al. reported that primary colorectal cancers, which showed *KRAS* or *BRAF* mutations, also frequently showed *RASSF2* methylation, and that inactivation of *RASSF2* enhanced KRAS-induced oncogenic transformation. RASSF2A exhibits several tumor suppressor properties, like inhibition of cell growth and induction of apoptosis [46,48]. In addition, patients with a methylated *RASSF2A* promoter presented a higher frequency of lymph node metastasis [48].

4.3. RASSF3

The *RASSF3* gene, which is located on chromosome 12 (locus 12q14.2), contains a RA domain and a SARAH domain (Figs. 1 and 2). RASSF3 shares almost 60% homology, at the amino acid level, with RASSF1 [51]. Its transcript is present in all tested normal tissues and tumor cell lines and no inactivating mutations in the coding region of RASSF3 in lung and breast tumors were found [51]. Overall only few data exist on RASSF3 and up to date no promoter methylation was detected in the few tumors studied [23,52].

4.4. RASSF4

The *RASSF4* gene is located on chromosome 10q11.21 (Fig. 2). RASSF4 harbors both a SARAH and a RA domain (Fig. 1) and has 25% homology to RASSF1A and 60% identity with RASSF2 [53]. *RASSF4* is transcribed from a CpG island region (Fig. 2) and frequently down-regulated by promoter methylation in human tumor cells and in several cancer cell lines, but broadly expressed in normal tissue [53]. RASSF4 shows tumor suppressor properties as indicated by growth inhibition and induction of apoptosis in human cancer cell lines [53]. In contrast, epigenetic inactivation of *RASSF4* in nasopharyngeal carcinoma is a rare event [54]. Eckfeld et al. stated that inactivating mutations were not detected in the coding sequence of RASSF4 [53]. Using human SNP microarray technology it was shown that in prostate cancer the locus 10q11.21 (amongst other loci) is affected by loss of heterozygosity [55]. Interestingly, in prostate cancer the *RASSF1A* promoter is often hypermethylated as well [56].

4.5. RASSF5

RASSF5, often called NORE1 (Novel Ras Effector 1), was the first member of the Ras-Association Domain Family to become characterized [2]. RASSF5 is up to 60% homologous to RASSF1 and the *RASSF5* gene is localized at 1q32.1 [51]. In renal collecting duct carcinoma the same region was earlier reported to be affected by LOH [57], whereas others rather found an amplification of the locus 1q32 [58–62]. By positional cloning, RASSF5 was identified as a breakpoint-spanning gene in a familial clear cell renal cell carcinoma [63]. RASSF5 exists in at least three isoforms, as a result of alternative splicing and differential promoter usage; termed RASSF5A (NORE1A), RASSF5B

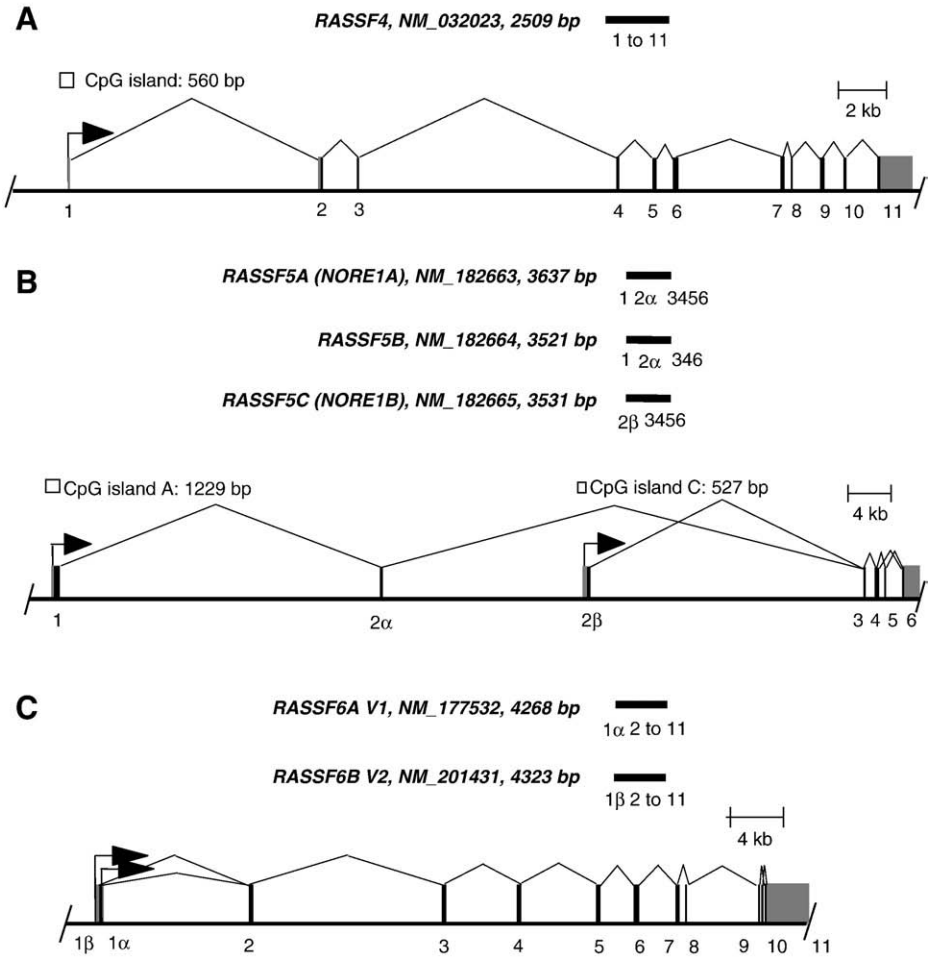

Fig. 3. Genomic arrangement of *RASSF4* at 10q11.21, *RASSF5* (*NORE1*) at 1q32.1 and *RASSF6* at 4q13. For details see legend of Fig. 2. (A) *RASSF4* codes for eleven exons. *RASSF4* is transcribed from a CpG island and its transcription start site is marked by an arrow. (B) *RASSF5* codes for seven exons termed 1, 2α , 2β , 3, 4, 5 and 6. The three isoforms RASSF5A (NORE1A), *RASSF5B* and *RASSF5C* (*NORE1B*) are shown. *RASSF5A* and *RASSF5C* are transcribed from two distinct CpG island promoters. (C) *RASSF6* codes for twelve exons termed 1α, 1β and 2 to 11. Two transcripts *RASSF6A* (Variant 1; V1) and *RASSF6B* (Variant 2, V2) are depicted. No CpG island was found in the promoter region.

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and RASSF5C (NORE1B) (Figs. 1 and 3). *RASSF5A* and *RASSF5C* are transcribed from separate CpG islands [51]. The domain structure of these two isoforms shows a RA domain as well as a SARAH domain, whereas the B isoform misses the SARAH domain due to exon skipping (Figs. 1 and 3). In RASSF5A and RASSF5B a C1 domain can also be found (Fig. 1). For clarity it has to be mentioned that RASSF5C is often also named NORE1B, however, both relate to the isoform that is transcribed from the downstream CpG island promoter (online: NCBI, Entrez Gene, 2008; InterProScan: http://www.ebi.ac.uk/ InterProScan/, 2008).

RASSF5A and RASSF5C were shown to be expressed in most normal tissues, but are down-regulated in various tumor cell lines though not in all studied [51,63–65]. Quite a few studies have investigated the methylation status of the *RASSF5* promoter with differing results. Tommasi et al. found no evidence for methylation in the *RASSF5C* CpG island of different tumors and stated that gene silencing due to promoter methylation of RASSF5 is not a common event in human primary tumors [51]. Chow et al. also found no aberrant methylation of *RASSF5A* in primary tumors [54]. A partial methylation was found in some nasopharyngeal carcinoma cell lines/xenografts, while expression of *RASSF5A* was found in all of these [54]. Nakamura et al. reported that *RASSF5A* methylation is an uncommon event in primary thyroid tumors [66] and also Foukakis et al. found no *RASSF5A* promotor methylation in thyroid tumors [67]. In gallbladder carcinomas, *RASSF5* was reported to be unmethylated [68]. Di Gioia et al. stated that they never found the *RASSF5A* gene epigenetically altered in hepatitic or non-hepatitic liver [69]. However, Macheiner et al. demonstrated that RASSF5C was epigenetically inactivated in 2/3 of the tested human hepatocellular carcinomas [70]. Hesson et al. showed that the *RASSF5A* promotor is hypermethylated in some primary tumors and tumor cell lines and RASSF5A, therefore, inactivated in a subset of cancers [71]. *RASSF5C* was reported to be unmethylated in gliomas, while *RASSF5A* showed methylation in glioma cell lines but not in primary tumors [52]. *RASSF5A* is frequently suppressed in pheochromocytoma and abdominal paraganglioma and its silencing was said to contribute to the transformed phenotype in these tumors [72]. Geli et al. found *RASSF5A* transcriptional suppression in neuroblastoma tumors and cell lines. Promotor methylation, however, was said to be not a common mechanism responsible for *RASSF5A* transcriptional suppression in this tumor type [73].

4.6. RASSF6

The *RASSF6* gene, which is located on chromosome 4q13.3, is predicted to be transcribed into two transcripts (online: NCBI, Entrez Gene, 2008, Figs. 1 and 3). The *RASSF6* transcripts differ in their first exons and, therefore, different translation start sites are predicted. RASSF6A, in comparison to the B isoform, has a shortened N-terminus (Fig. 1). RASSF6 contains a RA as well as a SARAH domain [74,75]. The *RASSF6* transcript could be detected in several tumor cell lines, but was often reduced in primary tumors [75,76]. Using *in silico* analysis, no CpG island was found in proximity to the first exons of *RASSF6* (Fig. 3); therefore, mechanisms of its down-regulation need to be further revealed (e.g. LOH). RASSF6 was able to inhibit the growth and promote apoptosis in specific tumor cell lines [75,76]. Taken together, the existing data suggest that RASSF6 may play a role in tumorigenesis.

4.7. RASSF7, RASSF8, RASSF9 and RASSF10

Only recently, four new members joined the Ras-Association Domain Family and were termed RASSF7, RASSF8, RASSF9 and RASSF10 (Fig. 1). Sherwood et al. reported that the RASSF members 7 to 10 represent the evolutionarily conserved so-called N-terminal RASSF group due to the N-terminal localization of their RA domains [77]. However these 4 members lack the SARAH domain otherwise present in RASSFs [77]. *RASSF7* is located at locus 11p15.5 and *RASSF8* at 12p12.3 (both Fig. 4). *RASSF9* is located at 12q21.31 and *RASSF10* at 11p15.2 (both Fig. 4). Interestingly only RASSF7, RASSF8 and RASSF10 are transcribed from CpG island regions.

Sherwood et al. demonstrated that *Xenopus* rassf7 is a centrosome-associated protein required for spindle formation and completing mitosis in the neural tube [77] and *RASSF7* was shown to be amongst the genes that discriminate islet cell tumors from normal human tissues [78]. Sherwood et al. stated that RASSF7 was found to be up-regulated 87-fold in tumors compared with normal tissues and concluded that this argues against RASSF7 having a tumor-suppressing role and raised the possibility that RASSF7 may even promote cancer formation [77]. Regarding RASSF8, no significant association between common SNPs in RASSF8 and lung cancer risk was found. However, *RASSF8* was found to be down-regulated in lung cancer and lung cancer cells exhibited a reduced growth rate in soft agar when transfected with RASSF8 [79,80]. Regarding to what is known about RASSF9 Alam et al. used a yeast-two-hybrid screen to identify novel partners for PAM (peptidylglycine α-amidating monooxygenase) and found RASSF9 (then named P-CIP1; *P*AM *C*OOH-terminal *i*nteractor *p*rotein 1) to be among these [81]. Chen et al. then cloned and characterized RASSF9 (P-CIP1) and found it highly conserved from rat to human (85% identity). They reported that it interacted with the cytosolic domain of wild type PAM-1 and that it associates with endosomes [82]. However the precise roles of RASSF7, RASSF8, RASSF9 and RASSF10 in tumorigenesis remain to be elucidated.

5. RASSFs and the microtubule network

The microtubule network functions as a cell scaffold spanning the cytoplasm and consisting of rigid but adjustable tubulin polymers. The network determines cell shape, takes part in cell motility, provides "tracks" for transport processes, functions as a scaffold for protein–protein interaction and compartmentalizes the cell. Furthermore, it is indispensable for a correct chromatid separation during mitosis. During interphase, microtubules exist in form of a network radiating from the MTOC (microtubule organizing center). During mitosis it reorganizes into two spindle networks radiating from two centrosomes. A correct chromatid separation is directly dependent on a functioning microtubule network [83–85].

5.1. RASSF1A

It was demonstrated that RASSF1A co-localizes with the microtubule network during interphase and is found at the spindles and centrosomes during mitosis [86,87]. RASSF1A binds to tubulin [31], thereby stabilizing microtubules [31,86,88,89] and regulating the mitotic progression. RASSF1A overexpression was shown to lead to an arrest at different stages the cell cycle: at metaphase [86], G1 [42], G2/M [90], G1 and G2/M [31] and at prometaphase [87]. The domain required for both microtubule association and stabilization was mapped to an amino-acid fragment from 120 to 288 [86] and another group showed this for the region 120 to 185 [88]. Dallol et al. reported two RASSF1A missense mutations, found in tumors at position C65R and R257Q, showed aberrant localization of RASSF1A and reported that these were not able to bind and stabilize microtubule [89]. However another study showed that the C65R mutant retains the ability to complex with microtubules [91]. Furthermore, it was reported that a common polymorphism at codon 133, which alters the microtubule association and stabilization domain of RASSF1A, is associated with an increase in the number of breast tumors [26]. Only recently, it was observed that RASSF1A localizes to the centrosome as part of the MST/WW45/LATS complex [92], further linking the isoform A to the microtubule network.

Interestingly, RASSF1A was also reported to interact with MAP1B (microtubule-associated protein 1B) and C19ORF5 (chromosome 19 open reading frames 5), both microtubule-associated proteins

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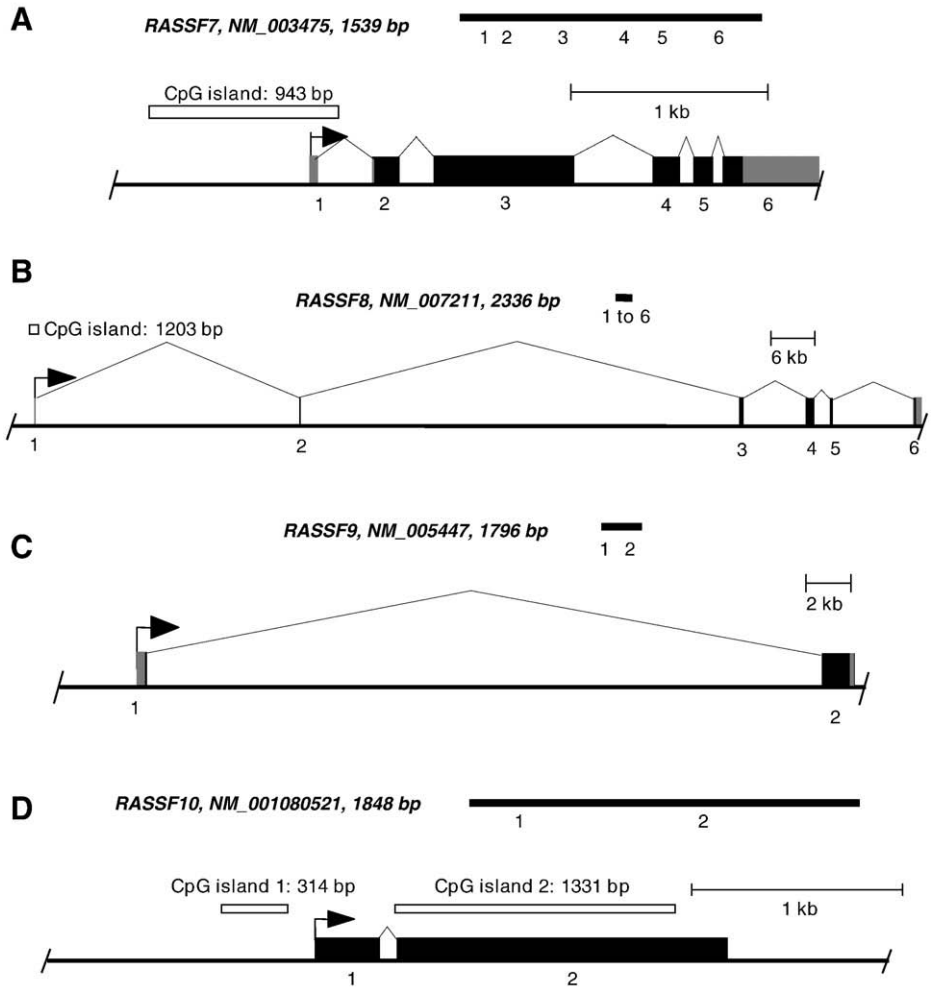

Fig. 4. Genomic arrangement of *RASSF7* at 11p15.5, *RASSF8* at 12p12.3, *RASSF9* at 12q21.31 and *RASSF10* at 11p15.2. For details see legend of Fig. 2. (A) *RASSF7* codes for six exons. Location and size of the CpG island was determined by CpG plot. (B) *RASSF8* encodes six exons. *RASSF8* is transcribed from a CpG island promoter. (C) *RASSF9* codes for two exons. No CpG island is found next to the transcriptional start site (arrow). (D) *RASSF10* encodes two exons. Two CpG islands are found at the *RASSF10* locus.

[89,93,94]. MAP1B is one of the major growth-associated and cytoskeletal proteins in neuronal and glial cells [95]. C19ORF5 is a hyperstabilized microtubule-specific binding protein of which accumulation causes mitochondrial aggregation and cell death [96]. Regarding C19ORF5, it was demonstrated that its knockdown led to mitotic abnormalities [97], that it localizes to centrosomes and it was stated that C19ORF5 is required for the recruitment of RASSF1A to the spindle poles [94,97]. Liu et al. reported that RASSF1A caused hyperstabilization of microtubules and the accumulation of C19ORF5 on them [96]. It was shown that RASSF1A is being phosphorylated within its RA domain (position 202/203) by Aurora-A during mitosis, and it was reported that this interfered with RASSF1A's ability to interact with microtubules and abolished its ability to induce M-phase cell cycle arrest [98]. Aurora-A overexpression also interferes with RASSF1A-mediated growth suppression [98]. Recently, Liu et al. showed that RASSF1A overexpression inhibits centrosome separation and demonstrated that RASSF1A interacts with the mitotic kinase Aurora-A. It was suggested that RASSF1A plays a role in the activation of Aurora-A, and it was proposed that RASSF1A may function as a scaffold for Aurora-A and its actual activator [99]. Verma et al. showed that RASSF1A is a substrate for protein kinase C (PKC) and that the tumor suppressor becomes phosphorylated by the kinase at positions S197 and S203. It was also reported that the phosphorylation mimicking double mutant of RASSF1A fails to modulate microtubule organization and also fails to cause the perinuclear collapse of the vimentin filaments [100]. In conclusion, RASSF1A's growth inhibitory function could at least partly be depending on its modified interaction with the microtubule network.

Foley et al. hypothesized that RASSF1A could be also linked to endosomal trafficking via interacting with tumor necrosis factor receptor 1 (TNF-R1) and stated that TNF-R1 internalization may be dependent on a stable microtubular network influenced by RASSF1A (for further detail on RASSF1A/MOAP-1/TNF-R1 see Section 8.1) [16].

5.2. RASSF1C

It was demonstrated that RASSF1C interacts with and stabilizes microtubules and, additionally, induces cell cycle arrest at G2/M, as described for RASSF1A [31]. In contrast, Song et al. could neither show an interaction of RASSF1C with microtubules, nor an effect on cell cycle distribution [87]. Vos et al. demonstrated that RASSF1C associated with microtubules, but was less effective at stabilizing them [88]. Liu et al. reported that RASSF1C co-localizes with microtubules and stabilizes these comparably with RASSF1A [96]. However, when coexpressed with C19ORF5, RASSF1C failed to associate with and promote hyperstabilization of microtubules and both RASSF1C and C19ORF5 remain dispersed in the cytosol. This difference to RASSF1A was said to be due to the short N-terminal sequence of RASSF1C rather than the unique features of RASSF1A [96].

Using mutation studies, the sequence of RASSF1A and RASSF1C, which is responsible for interaction and stabilization of microtubules,

was shown to encompass the C-terminal 6 residues (AEIEQK) of the W125-K138 ATM consensus sequence downstream of serine 131 [96]. Kumari et al. reported that RASSF1C localizes to the microtubule network, but also found that it interacts with importin-alpha and is transported into the nucleus [101].

5.3. RASSF5

RASSF5 was shown to be a centrosomal protein capable of microtubule binding, for which the RA-domain is essential [90]. Furthermore, it was suggested that microtubule binding is required for RASSF5's growth suppressive and tumor suppressive activities, which are transduced through inhibiting the ERK signaling pathway [90].

5.4. RASSF7

Consistent with a role in regulating spindle microtubules, rassf7 (*Xenopus*) was found to localize to centrosomes in a microtubule-dependent manner and its knockdown led to cells failing to form a spindle and arrest in mitosis [77].

6. RASSFs and the protooncogene *RAS*

The membrane-bound small GTPase RAS has a central role in regulating mammalian cell growth. RAS conveys growth stimuli from the cell surface to the inner cell as a proproliferative signal of the RAS-MAP-kinase pathway (serine/threonine kinase cascade). Following RAS activation, a transcriptional activation of e.g. Cyclin D1 and other factors is mediated [102], by which this mechanism promotes cell proliferation. The same effect can be observed though RAS interacting with the PI3K-pathway, thereby promoting an additional antiapoptotic effect. However, there are also proapoptotic and cell cycle inhibiting functions of RAS as described in the literature, that serve as a protective mechanism against a deregulated RAS-pathway (e.g. constitutively active RAS) [103].

Proteins that bind RAS (in its active state with bound GTP) are called Ras effectors and contain either Ras-binding or Ras-association domains [104,105]. The Ras-association (RA) domain is a characteristic feature of RASSFs and several studies investigated the ability of RASSFs to interact with different RAS family proteins. The RAS family comprises at least 21 members such as HRAS, KRAS, NRAS and RRAS [106]. Therefore, an interaction of RASSFs with the protooncogene RAS could indicate their potential role as negative RAS-effectors that antagonize cancer development and support their classification as tumor suppressors.

A preferential ability of **RASSF1A** to heterodimerize with RASSF5 and thereby associate with Ras-like GTPases was reported, whereas RASSF1A alone binds only weakly to RAS [20]. RASSF1 exhibits relatively low affinity for Ras-like GTPases but was said to possibly associate with Ras-GTP indirectly [107]. The RASSF1A–MST complex binds the scaffold protein CNK1, through which these also participate in the proapoptotic signaling initiated by active RAS (for further details see Section 8) [108]. Armesilla et al. further linked RASSF1A to RAS signaling and showed an interaction of PMCA4b (plasma membrane Ca^{2+} pump 4b) with RASSF1A significantly inhibited the EGF-dependent activation of the mitogen-activated protein kinase ERK [109].

And additionally in some tumor types a reciprocal relationship between activated KRAS and RASSF1A hypermethylation could be observed [110–112].

For the other major isoform **RASSF1C** it was shown that it interacts with RAS in a GTP-dependent manner to mediate apoptosis [43]. Overexpression of **RASSF1C** with members of the RAS-family showed an interaction with RRAS3/ M-RAS and a weak interaction with NRAS and KRAS [106]. Kitagawa et al. showed that RASSF1C binds the active form of HRAS and the two proteins cooperate to activate the SAPK/JNK pathway [113].

RASSF2 binds directly to KRAS in a GTP-dependent manner via its RA domain. However, RASSF2 only weakly interacts with HRAS. It was shown that a RASSF2-mediated cell death is enhanced by KRAS and appears to involve both apoptosis and cell cycle arrest [46]. RASSF2 interacts with members of the RAS family in a pulldown study, in which it displays with RRAS3 the strongest and with RRAS a weakly detectable interaction [106].

In different tumor entities, *RASSF2* promoter hypermethylation was positively correlated with mutations found in *KRAS* and *BRAF* [49,50,114]; however, for lung cancer and lung cancer cell lines no correlation between *KRAS* mutation and promoter hypermethylation of *RASSF2* could be observed [115]. Multiple logistic analyses showed that lymphatic invasion and *RASSF2* methylation with *KRAS* and *BRAF* mutations were independent risk factors for venous invasion in pT1 colorectal carcinomas [114]. Furthermore, inactivation of *RASSF2* enhanced KRAS-induced oncogenic transformation [49].

RASSF4 interacts with activated KRAS in a GTP-dependent manner via its association domain and it was reported that activated KRAS stimulates RASSF4-mediated cell death [53]. RASSF4 binds to RAS family members in a pulldown study; e.g. NRAS, KRAS, RRAS and RRAS3 [106].

RASSF5 binds to RAS in a GTP-dependent manner and the interaction requires an intact Ras-association domain [2]. RASSF5A binds preferentially to the GTP-charged forms of RAS, RAP-1, and several other RAS subfamily GTPases with high affinity [107]. Ikeda et al. showed that RASSF5 binds various member of the RAS family [75]. RASSF5 becomes associated with RAS *in situ* following activation of epidermal growth factor receptor in COS-7 and in KB (human oral carcinoma) cells [2]. On the one hand, it was reported that *RAS* mutations and RASSF5A down-regulation were mutually exclusive [67], but on the contrary no correlation was observed between RASSF5 expression and the presence or absence of a *RAS* mutation [65]. It was also shown that the RASSF5-mediated growth inhibition is RAS-dependent [65]. However, another group found that Rassf5 suppresses the growth of some tumor cell lines through yet unidentified effectors, independent of Ras-like proteins [64]. Khokhlatchev et al. reported that active RAS binds the RASSF5–MST1 complex and that the RASSF5–MST1 complex is a novel Ras effector unit that mediates the apoptotic effect of KiRasG12V [116]. Ortiz-Vega et al. reported that a heterodimerization of RASSF5 with RASSF1A is necessary for an association of RASSF1A with RasG12V [20]. A pulldown study reported that RASSF5 binds to several RAS family members [106]. Kumari et al. reported that RASSF5 binds strongly to KRAS but weakly to both NRAS and HRAS, though data were not shown [101]. Ishiguro et al. indicated that RASSF5C participates in the recruitment of activated RAS into the immune synapse, and is necessary for optimal RAS signaling in response to T cell receptor activation [117].

RASSF6's binding of RAS remains to be further investigated since inconsistent reports exist. It was shown that although RASSF6 has an RA domain, it does not bind RAS under the condition that RASSF5 binds these RAS proteins [75]. Allen et al. reported that RASSF6 binds activated KRAS in a GTP-dependent manner and through its RA-domain. Furthermore, it was stated that the exogenous expression of RASSF6 promoted apoptosis and RASSF6 synergized with activated KRAS to induce cell death. RASSF6 was also reported to inhibit the survival of specific tumor cell lines [76].

Up to now, the only connection of **RASSF7**, **RASSF8** and **RASSF10** to RAS is, that these *RASSF* genes encode Ras-association domains and are located in proximity to the *HRAS1*, *KRAS2* and *RRAS2* genes [77,80,118], indication a common evolutionary origin of these RASSF members.

7. RASSF and the Hippo pathway

In *Drosophila*, the Hippo (Hpo) signaling pathway is involved in tumor suppression and organ size control [119]. The Hippo pathway

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promotes proper exit from the cell cycle and apoptosis during development [22,119]. Among the proteins associated with this pathway, Salvador (Sav) interacts with Hpo and Wts and, therefore, acts as a scaffold protein for these. Sav shows tumor suppressor properties and its human orthologue is WW45. Warts (Wts) is a protein kinase and tumor suppressor, and its mammalian orthologues are LATS1 and LATS2 (large tumor suppressor). Wts itself becomes phosphorylated and activated by Hpo. Upon activation, Wts phosphorylates and inhibits Yorkie, a transcriptional coactivator that regulates the expression of genes associated with apoptosis (*diap1, drosophila inhibitor of apoptosis-1*) and cell proliferation (*cyclin E*). Hippo (Hpo) is a proapoptotic protein kinase and its human orthologues are MST1 and MST2 (mammalian sterile 20-like kinase-1 and -2). Through pathways that are not fully understood yet, Hpo senses membrane associated signals and activates Wts through phosphorylation. Taken together Hpo activates Wts via phosphorylation and Sav serves as a scaffold for these two [119,120]. It was shown that the single *Drosophila* orthologue of the human RASSF proteins restricts Hpo activity by competing with Sav for binding to Hpo [121]. It has been proposed that the interactions between Sav, Rassf and Hpo are accomplished by the protein–protein binding domain termed SARAH (Sav–RASSF–Hpo) [19].

Various studies show the orthologous human pathway to be involved in tumorigenesis [reviewed in 119, 120]. Regarding MST1 (homologue of Hpo) it is known that it becomes activated through caspase-dependent cleavage following death receptor activation [122]. The cleaved fragment then localizes from the cytoplasm to the nucleus, where it induces apoptosis [122] by chromatin condensation through activation of the c-Jun N-terminal kinase pathway [123]. On the other hand, MST1 phosphorylation leads to its autoactivation [122]. Membrane localization of MST1 enhances its proapoptotic effect [116]. According to Avruch et al., each of the RASSF polypeptides binds to MST1 and MST2 through the SARAH domains of each partner [107]. The SARAH domain is a characteristic feature of MST1, WW45 and RASSFs.

Regarding the relationship between human RASSFs and members of the orthologous human Hippo pathway, it was reported that **RASSF1**A/C interact with MST1 [116,124]. Praskova showed *in vitro* that the MST1 kinase autoactivation through phosphorylation is inhibited by coexpression of RASSF1A, RASSF1C, RASSF5A or RASSF5C. Regarding RASSF1 and RASSF5 it was proposed that these direct MST1 to sites of activation and perhaps co-localization with endogenous substrates [124]. Another group later found that RASSF1A is required for full activation of MST1 during Fas-induced apoptosis and, as a result, enhances MST1-mediated apoptosis *in vivo* [122]. RASSF1A and MST1 were found to co-localize at microtubules during the cell cycle [122]. In addition, it was found that human RASSF1A is part of the MST2/WW45/LATS1 complex (mammalian Hippo complex) at the centrosome, and it was shown that defects in this pathway may lead to abnormal mitosis caused by cytokinesis failure and failure to induce apoptosis [92]. A mitotic role for MST1 and MST2 was also suggested recently [125]. It was proposed that RASSF1A induces apoptosis via binding to MST2, which is released from inhibition by RAF-1 following Fas activation [126]. Subsequently MST2, activated by the interaction with RASSF1A, binds to LATS, and YAP1 becomes phosphorylated by LATS and dissociates from LATS. YAP1 then translocates to the nucleus and forms a complex with p73 ultimately leading to the transcription of *PUMA*, which initiates apoptosis [126].

RASSF2 was shown to interact with MST1 using a yeast-two-hybrid system [23,124] and demonstrated to bind to MST1 [116]. **RASSF3** was also reported to bind MST1 in a yeast-two-hybrid system [124] and **RASSF4** bound to MST1 when both were overexpressed, though data were not presented [53]. **RASSF5** binds to MST1 (and MST2, though data not shown), activated RAS binds the RASSF5–MST1 complex *in vivo* and that complex is able to mediate the apoptotic effects of constitutively active RAS (KiRasG12V). RASSF5 was also said to interact with MST2 [116].

8. RASSFs and apoptosis

Apoptosis is the programmed cell death that can be initiated by intrinsic as well as extrinsic signals, which ultimately leads to the destruction of the cell following a defined cascade. While the outer membrane of the cell is still intact, in contrast to the inflammatory cell death (necrosis), the inner of the cell undergoes nuclear and cytoplasmic condensation. Finally, the membrane enclosed remains are incorporated by phagocytes or neighboring cells. Besides maintenance of tissue homeostasis, apoptosis is also implicated in human diseases e.g. the loss of the ability to induce apoptosis is related to cancer development. A promising strategy in cancer therapy is re-establishing the ability of tumor cells to undergo apoptosis and, therefore, elucidating the cellular processes through which the RASSF tumor suppressors take part in apoptosis is of major importance [127,128].

A central role in apoptosis is played by mitochondria, a double membraned cell organelle. During intrinsic apoptosis various proapoptotic proteins are released from the intermembrane space of mitochondria, e.g. Cytochrome C. In complex with other cytosolic proteins the apoptotic signal is then further passed on. The protein BAX, which is referred to below, is involved in the permeabilization of the outer mitochondrial membrane leading to the release of Cytochrome C. Although there are many more factors playing a role in apoptosis [127,128] the description of apoptosis was kept brief in this review for the purposes of clarity.

8.1. RASSF1

In literature there are different apoptotic pathways that RASSF1 was said to be involved in. It was reported by Baksh et al. that RASSF1A is required for death receptor-induced BAX conformational change and apoptosis. After receptor stimulation RASSF1A and the modulator of apoptosis-1 (MOAP-1 or MAP-1; a BH3-like protein) are recruited to the membrane and form a complex. When RASSF1A binds MOAP-1, this releases MOAP-1's intramolecular inhibition, and MOAP-1 is then able to associate with BAX [129]. It was further shown by Vos et al. that the binding of RASSF1A to MOAP-1 is enhanced in the presence of activated KRAS. RASSF1A activates BAX through MOAP-1 and activated KRAS, RASSF1A and MOAP-1 synergize to induce BAX activation and cell death [91]. Foley et al. found RASSF1A to be involved in the death receptor-dependent apoptosis pathway (extrinsic pathway). Upon tumor necrosis factor alpha (TNFα) stimulation, MOAP-1 associates with the TNF receptor 1, RASSF1A is recruited to this complex and RASSF1A homodimerization is lost prior to the recruitment. Therefore, RASSF1A is linked to death receptor-dependent apoptosis [16] and results from Reu et al. identified RASSF1A as regulated by interferons and participating in IFN-induced apoptosis at least in part by sensitization to TRAIL [130].

Expressing RASSF1A in breast cancer cells lacking endogenous RASSF1A led to a death receptor-dependent induction of apoptosis [129]. However, it was found that RASSF1A itself provokes little or no apoptosis in human embryonic kidney 293 cells, whereas coexpression with CNK1 substantially augments CNK1-induced cell death. CNK1 (connector enhancer of KSR) is a multidomain scaffold protein and is required for RAS activation of RAF kinase. CNK1 was found to interact with MST1 and MST2 and CNK1-induced cell death requires the MST kinase. Deletion of the MST1 carboxyl-terminal segment that mediates its binding to RASSF1A and C eliminates the association of MST1 with CNK1. In conclusion CNK1 binds RASSF1A and promotes apoptosis when overexpressed through a pathway that depends on RASSF1A and MST1/2. In comparison RASSF1C does not alter the extent of CNK1-induced apoptosis, although being able to bind CNK1, MST1 and MST2 [108].

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Vos et al. demonstrated that RASSF1C overexpression induces apoptosis and that activated RAS enhances and dominant negative RAS inhibits this effect [43]. It was reported that RASSF1C binds to DAXX (involved in apoptosis and transcriptional repression) in nuclear protein complexes, called promyelocytic leukemia-nuclear bodies (PML-NBs) [113]. PML-NBs are dynamic sensors of cellular stress and DNA damage leads to their disassembly, whereas DAXX becomes degraded and RASSF1C is released into the cytoplasm. RASSF1C then translocates to microtubules and participates in the activation of SAPK/JNK. The SAPK/JNK signaling pathway, activated after DNA damage, plays a crucial role in the regulation of gene expression leading to apoptosis [113]. The JNK kinase was implicated to play an important role in oncogenic transformation through the apoptotic elimination of transformed cells [131]. RASSF1C binds activated HRAS and cooperate to activate the SAPK/JNK pathway [113]. Oncogenic RAS activates the SAPK/JNK pathway [132]. Furthermore, it was reported that dominant negative HRAS and dominant negative MST1 inhibit DNA damage-induced SAPK/JNK activation in HeLa cells. Therefore, it was reasoned that RASSF1C may positively regulate RAS–RASSF5–MST1 signaling and thus activates SAPK/JNK [113]. Regarding RASSF1A it was shown that it inhibits the SAPK/JNK pathway and, therefore, the growth of lung cancer cells. It was reported that RASSF1A suppresses the phosphorylation of JNK [133]. RASSF1A was further linked to cell death through interacting with the microtubule-associated protein C19ORF5 [93,96].

In summary, RASSF1A was reported to induce apoptosis via interaction with MST1, RAS, RASSF5, CNK1, MOAP-1 and C19ORF5. RASSF1C is linked to apoptosis through the SAPK/JNK signaling pathway, RAS and MST1. It has to be kept in mind, however, that much of the data linking RASSF1A to cell death pathways were obtained under conditions where RASSF1A was overexpressed, and this may not reflect a situation that is biologically relevant. Activating signals that may provide a physiological input into MST-dependent pro-apoptotic pathways or any other RASSF1A-dependent apoptosis pathways are currently unknown.

8.2. RASSF2

RASSF2 was reported to promote apoptosis [46,49]. Coexpression of activated KRAS and RASSF2 led to the observation that KRAS enhances RASSF2-mediated growth inhibition [46]. Akino et al. showed that inactivation of RASSF2 enhances KRAS-mediated cell transformation. Furthermore, it was shown that exogenous RASSF2A leads to disruption of the actin stress fiber network and to an increase in cortical actin. In addition it was stated that RASSF2A has a role in the suppression of the RAS-Rho pathway [49]. The Rho family is involved in regulating cell morphology and invasiveness through interacting with the actin skeleton and Rho itself can be indirectly activated by RAS. These observations have led to the conclusion that RASSF2 is involved in actin cytoskeleton organization and the hypothesis that RASSF2 induces a suspension-depended apoptosis, so-called anoikis [49].

8.3. RASSF4

Eckfeld et al. reported that RASSF4 binds RAS, and that overexpression of RASSF4 induces RAS-dependent apoptosis in 293-T cells and inhibits the growth of human tumor cell lines. The observed growth inhibition is enhanced by the addition of a RAS CAAX membrane localization signal to the COOH terminus of RASSF4 [53]. An established method of activating Ras effectors is to add a COOH-terminal CAAX membrane localization sequence to them [134]. Eckfeld et al. also reported that RASSF4 interacted with MST1, though data were not presented. It was, therefore, stated that just like RASSF5, which exhibits its proapoptotic effects through binding MST1, MST1 may also play a role in the function of RASSF4 [53].

8.4. RASSF5

Khokhlatchev et al. reported to have identified a novel pathway by which active KiRAS initiates apoptosis through the direct recruitment of its putative effector RASSF5, which is stably associated with the proapoptotic kinase MST1. Therefore, it was demonstrated that RASSF5 binds MST1 and that these form a complex *in vivo*, with which active RAS interacts after serum stimulation. It was shown that membrane recruitment of MST1 promotes its apoptotic action e.g. by coexpression with a membrane targeted variant of RASSF5. Active RAS was demonstrated to promote apoptosis through a RASSF5–MST1 complex [116]. In different mammalian cells it was reported that overexpressing RASSF5 alone however does not induce apoptosis [64,116] or only led to a small increase in apoptosis in NIH3T3 cells [116]. It was suggested by Aoyama et al. that the RASSF5 growth inhibition is independent of its ability to bind to activated RAS-like GTPases and to the MST1/2 kinases, and is primarily based upon the induction of cell cycle delay rather than apoptosis [64].

Regarding the proapoptotic kinase MST1 and RASSF5 it was shown that the autoactivation of MST1 through phosphorylation is inhibited by coexpression of RASSF5 [124]. For further details on the kinase MST, its function and the pathway that MST is involved in see Section 7. Vos et al. reported that Maxp1, the rat orthologue of RASSF5, was able to bind RAS in a GTP-dependent manner. In addition, it was demonstrated that rat Rassf5 inhibits cell growth in a RAS-dependent manner and that the growth inhibition is apoptotic in nature [65]. However, Moshnikova et al. found that cells reexpressing RASSF5A grew at the same rate as parental cells and showed no changes in cell cycle distribution. Furthermore, it was reported that no cells with a DNA content less than G0/G1 were observed, though data for the latter were not shown. It was suggested that, based on their studies, RASSF5A alone does not induce apoptosis, but it was said that RASSF5 is a cytoskeletal protein that suppresses cell growth by inhibition of the ERK pathway [90]. In contrast, Geli et al. found that transient RASSF5 expression in human neuroblastoma SK-N-BE cells enhances apoptosis and delays cell cycle progression [73]. Recently, Park et al. reported that the nuclear export of RASSF5A via its NES is involved in the RASSF5A-mediated induction of apoptosis [135].

8.5. RASSF6

It was reported that RASSF6 induces apoptosis via both caspase-dependent and caspase-independent pathways [75] and it was shown that RASSF6 synergizes with KRAS to induce cell death [76]. In contrast, it was found by another group that RASSF6 did not bind RAS proteins [75]. Furthermore, it was shown that RASSF6 immunoprecipitates with MOAP-1 (modulator of apoptosis-1), and it was observed that the interaction appeared to be enhanced in the presence of activated KRAS; therefore, it was suggested that this might serve as a mechanism by which RAS activates the proapoptotic effects of RASSF6 [76].

8.6. RASSF7

It was reported that knockdown of rassf7 (*Xenopus*) triggered nuclear fragmentation and apoptosis and from this and further experiments the possibility was raised that RASSF7 might promote cancer formation, in contrast to the tumor suppressing properties that several mammalian members of the family exhibit [77].

9. RASSFs and cell cycle regulation

Shivakumar et al. suggested that **RASSF1A** negatively regulates accumulation of endogenous Cyclin D1 through a posttranscriptional mechanism leading to inhibition of the cell cycle progression [136]. It was reported that transfection of RASSF1A into A549 cells induces G1

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cell cycle arrest and down-regulates Cyclin D1 expression [42]. Deng et al. showed that RASSF1A overexpression in a gastric carcinoma cell line led to inhibition of cell growth with G1 arrest. Furthermore, they found that RASSF1A overexpression inhibited AP-1 activity, and down-regulated c-Fos, but not c-Jun. The Activator protein-1 (AP-1) is a transcription factor that consists either of a Jun–Jun homodimer or of a Jun–Fos heterodimer [137]. AP-1 is involved in cell proliferation, differentiation and apoptosis [138]. It was also reported that Cyclin D1, a target of AP-1, is down-regulated after ectopic RASSF1A expression. Therefore, it was presumed that RASSF1A induces a G1 cell cycle arrest in a gastric carcinoma cell line by down-regulating Cyclin D1 through inhibition of AP-1 activity [137].

Recently, it was shown that SKP2, an oncogenic subunit of an ubiquitin ligase complex and a key regulator of S-phase entry, promotes the degradation of RASSF1A at the G1/S transition of the cell cycle. It was reported that this mechanism requires the phosphorylation of RASSF1A on serine 203 by Cyclin D-CDK4 *(cyclin dependent kinase 4)*. A delay of the cell cycle progression (G1/S phase) was observed when serine 203 in RASSF1A was mutated to alanine and an enforced expression of SKP2 abolished the RASSF1A inhibition of cell proliferation. It was found that the delay in G1/S progression after SKP2 removal is normalized by depletion of RASSF1A [139]. However, in our laboratory, we were not able to detect cell cycle-dependent fluctuations in the level of the RASSF1A protein (L. Liu and G.P. Pfeifer, unpublished results).

It was reported that RASSF1A, through interacting with CDC20 during early prometaphase, is able to inhibit the formation of the APC/C-CDC20 complex *(anaphase-promoting complex/ cyclosom-cell division cycle 20 homolog)* [87,140]. The APC/C complex is part of the ubiquitin-conjugation-system, which marks proteins for the degradation in the proteasome. Through inhibition of this complex, Cyclin A and B do not become proteasomally degraded. In addition, an inactive APC/C-CDC20 is not able to degrade the Separase-inhibitor, and, therefore, Separase is not able to separate the chromatids. However, our own groups did not see an interaction of RASSF1A with CDC20 [141].

Wang et al. proposed from their study a model for ID-1 *(Inhibitor of DNA binding/differential-1)* and the involvement of RASSF1A in mitosis. ID-1 interacts with CDC20 *(cell division cycle protein 20)* and RASSF1A during early mitosis, that leads to enhanced APC(CDC20) activity and, therefore, to Cyclin B1/securin degradation and premature mitosis. Whereas during late mitosis ID-1 binds to CDH1 *(CDC20 homolog 1)*, disrupting the interaction of CDH1 with APC and, therefore, suppressing APC(CDH1) activity [142].

Vos et al. demonstrated that **RASSF2** overexpressing cells consistently show a decrease in the G2/M phase of the cell cycle, suggesting that the cells tend to arrest in the G0/G1 phase [46].

Sherwood et al. reported that **rassf7** (*Xenopus*) knockdown led to cells, which failed to form a spindle and arrest in mitosis [77].

10. RASSFs and other pathways

Dallol et al. reported that RASSF1A is involved in controlling cell migration [143]. Studies found that RASSF5C (referred to as RAPL/NORE1B) regulates lymphocyte adhesion [144], is a regulator of immune cell trafficking and essential for immuno-surveillance [145]. RASSF5C was further linked to lymphocyte polarity and adhesion [146], T cell migration [147] and directional migration of vascular endothelial cells [148]. In 2005, details on RASSF5C and the regulation of immune cell adhesion and migration were reviewed [149]. Ishiguro et al. proposed a model in which RASSF5C modulates RAS signaling outputs triggered by T cell receptor (TCR) stimulation via recruiting active RAS to the plasma membrane and by contributing to the localization of Carma1, an essential lipid raft-associated regulator of TCR-induced NFκB activation [117]. Allen et al. reported that RASSF6 suppresses the NFκB pathway and proposed that RASSF6 may play a role in dictating the degree of inflammatory response to the respiratory syncytial virus [76].

Kumari et al. linked several members of the Ras-Association Domain Family to nuclear transport via either importin-alpha or -beta, through the identification of transferable arginine-rich localization signals within the family members RASSF1C, 2 and 5. Furthermore, it was shown that after overexpression in Cos-7 cells RASSF5 localizes both in the nucleus and the nucleolus in contrast to RASSF1C, which is found in addition to its microtubule localization also in the nucleus, and RASSF2, which is found in the nucleus but not the nucleolus.

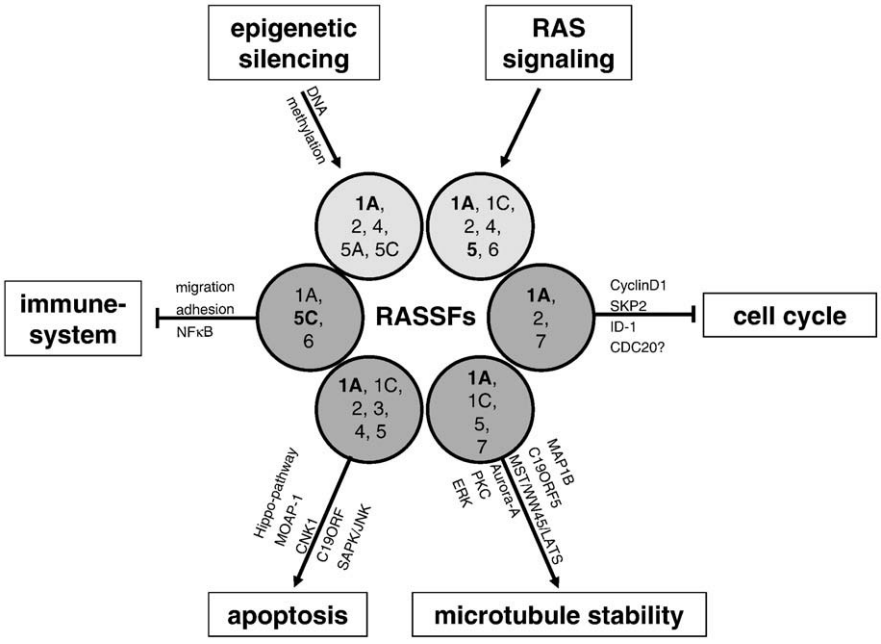

Fig. 5. Overview of the manifold RASSF functions. Epigenetic silencing and RAS signaling are shown to contribute to Ras-Association Domain Family regulation. RASSFs (abbr. 1 to 7) influence the listed pathways, e.g. microtubule stability, cell cycle, apoptosis, immune system and nuclear transport. The main pathways according to publications are shown and in bold the main family members are depicted.

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Additionally, the cell growth inhibitory activity of RASSF5 was said to be dependent on its nuclear localization [101]. Vos et al. further implicated RASSF1A in genomic instability. RASSF1A and RASSF1C were said to block activated RAS-induced genomic instability. A point mutant of RASSF1C, identified in human tumors, was found severely defective for stabilizing tubulin and unable to block the genomic destabilizing effects of RAS [88]. Two RASSF members were linked to the modulation of the β-Catenin/Wnt signaling pathway. Estrabaud et al. postulated that RASSF1C may be involved in tumorigenesis through inhibition of β-Catenin degradation, and found that RASSF1A and -1C had opposite effects on the β-Catenin degradation. They suggested that RASSF1C expressed in absence of RASSF1A could play a role in tumorigenesis [150]. Data from knockout mice demonstrated a cooperation between the inactivation of *Rassf1a* and *Apc* that resulted in an accelerated intestinal tumorigenesis, with adenomas showing an increased nuclear accumulation of β-catenin, supporting a mechanistic link via loss of the known interaction of *Rassf1* with β-TrCP that usually mediates degradation of β-catenin [34].

For further reading especially on the areas that were covered only in short in this review, as for the promoter methylation of the RASSF members, we recommend the following reviews on the Ras-Association Domain Family: "The tumor suppressor RASSF1A in human carcinogenesis"; "Evaluation of the 3p21.3 tumour-suppressor gene cluster"; "The Ras-association domain family (RASSF) members and their role in human tumourigenesis" and "The RASSF1A tumor suppressor" [13,151–153].

11. Conclusion

As summarized in this review and shown in Fig. 5, the following Ras-Association Domain Family members were already shown to exhibit tumor suppressing properties: RASSF1A, RASSF2, RASSF4, RASSF5 and RASSF6 (for refs. see above). Since the initial characterization of the RASSF family, various members have been added, and recently RASSF7 to RASSF10 were described and named the N-terminal RASSF family [77].

Among all RASSF family members, the tumor suppressor RASSF1A remains best studied. Rassf1a knockout mice are tumor-prone, but animal models for other Rassf family members have not been reported yet [except for the RASSF5C/RAPL/NORE1B knockout]. RASSF1A is the most frequently inactivated RASSF family member in human tumors and shows an extensive hypermethylation pattern of its promoter in various tumor types, which leads to inhibition of its expression (Fig. 5). At a functional level, much remains to be learned about all RASSF family members. In particular, it remains unclear which of the N-terminal RASSF proteins interact with which of the activated RAS family members. Moreover, the RASSF proteins may have functions that are completely independent of binding to RAS proteins, since computer-based prediction of this domain may not truly reflect its biological function as a Ras-association domain. It will be important to characterize the exact biochemical function of each family member. To date we do not even know how the family members with C-terminal and N-terminal RA domains, respectively, differ from each other in general.

Recent data point to a role of RASSF family members in cell cycle regulation and/or apoptosis (Fig. 5). As mentioned, many of the results published so far must be interpreted with caution inasmuch as they are based on overexpression studies. Interesting new data and directions will most likely arise from the recent discovery that RASSF1A is a component of the mammalian Hippo pathway (Fig. 5) [92]. Other RASSF family members may be involved in this pathway, as these were already demonstrated to interact with MST (Hippo) kinases. However, it is completely unknown how this pathway is regulated in mammalian systems. This is true for the upstream input signals as well as for the downstream components that ultimately mediate Hippo pathway functions. An important area of investigation will be to discriminate how the RASSF pathway bifurcates into a cell cycle regulatory system and a cell death-inducing system. Ultimately, we may understand the contribution of the epigenetic inactivation of *RASSF* genes to tumorigenesis.

Acknowledgements

The research in Reinhard Dammann's laboratory is funded by the Deutsche Krebshilfe and the Deutsche Forschungsgemeinschaft (DFG DA552). Gerd Pfeifer's work is supported by a grant of the University of California Tobacco-Related Disease Research Program (17-RT-0116).

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Please cite this article as: A.M. Richter et al., The RASSF proteins in cancer; from epigenetic silencing to functional characterization, Biochim. Biophys. Acta (2009), doi:10.1016/j.bbcan.2009.03.004

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Please cite this article as: A.M. Richter et al., The RASSF proteins in cancer; from epigenetic silencing to functional characterization, Biochim. Biophys. Acta (2009), doi:10.1016/j.bbcan.2009.03.004

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