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REVIEW

Tumor suppressor C-RASSF proteins

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Abstract

Human genome has ten genes that are collectedly called Ras association domain family (*RASSF*). *RASSF* is composed of two subclasses, *C-RASSF* and *N-RASSF*. Both *N-RASSF* and *C-RASSF* encode Ras association domain-containing proteins and are frequently suppressed by DNA hypermethylation in human cancers. However, *C-RASSF* and *N-RASSF* are quite different. Six C-RASSF proteins (RASSF1–6) are characterized by a C-terminal coiled-coil motif named Salvador/RASSF/Hippo domain, while four N-RASSF proteins (RASSF7–10) lack it. C-RASSF proteins interact with mammalian Ste20-like kinases—the core kinases of the tumor suppressor Hippo pathway—and cross-talk with this pathway. Some of them share the same interacting molecules such as MDM2 and exert the tumor suppressor role in similar manners. Nevertheless, each C-RASSF protein has distinct characters. In this review, we summarize our current knowledge of how C-RASSF proteins play tumor suppressor roles and discuss the similarities and differences among C-RASSF proteins.

Keywords Apoptosis · Cell cycle · Hippo pathway · Ras · Tumor suppressor

Abbreviations

APC/C	Anaphase-promoting complex
ARF6	Adenosine diphosphate ribosylation factor 6
ATM	Ataxia telangiectasia mutated
CNKR1	Connector enhancer of kinase suppressor Ras 1
ER	Endoplasmic reticulum
HIPK2	Homeodomain-interacting protein kinase 2
JNK	c-Jun N-terminal kinase
LATS	Large tumor suppressor kinase
MAGI	Membrane-associated guanylate kinase inverted
MOAP1	Modulator of apoptosis 1
MST	Mammalian Ste20-like kinase
NORE	Novel Ras effector
PAR-4	Prostate apoptosis response protein-4
PKA	Protein kinase A
PKC	Protein kinase C
RA	Ras association
RCC1	Regulator of chromosome condensation 1
SARAH	Salvador/RASSF/Hippo
STRIPAK	Striatin-interacting phosphatase and kinase
TAZ	Transcriptional coactivator with PDZ-binding motif
TEAD	TEA domain transcription factor
TNF-α	Tumor necrosis factor-α
TRAIL	Tumor necrosis factor-like apoptosis-inducing ligand
XPA	Xeroderma pigmentosum A
YAP1	Yes-associated protein 1

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Introduction

The human genome contains ten genes designated as Ras association (RA) domain family (*RASSF*) members [1, 2]. These genes encode proteins with one RA domain. Among them, RASSF1–6 harbor a coiled-coil motif in the C-terminal region (Fig. 1). As similar sequences are detected in the *Drosophila* proteins Salvador and Hippo, this motif is named the Salvador/RASSF/Hippo (SARAH) domain. RASSF7–10 lack this motif [3]. The SARAH domain is involved in cross-talk with the tumor suppressor Hippo

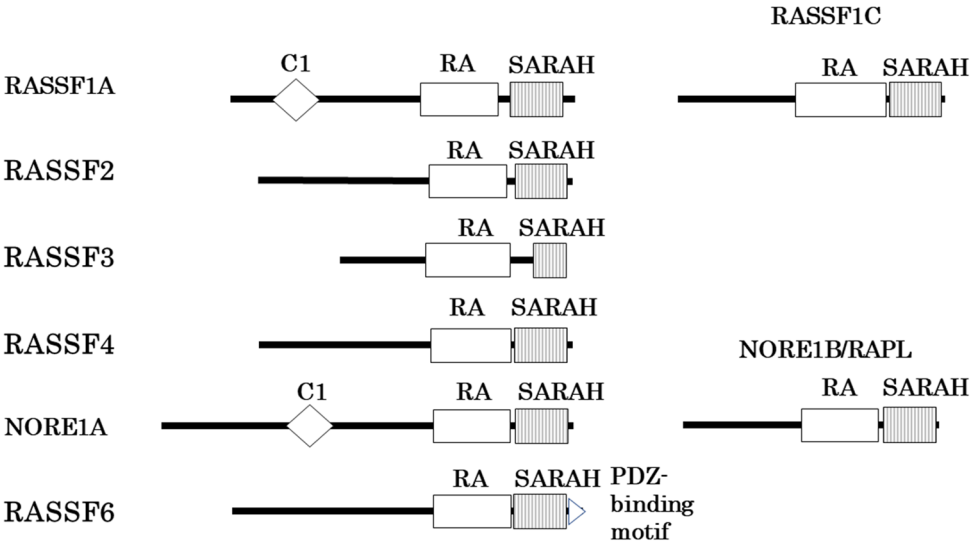

Fig. 1 Mammalian C-RASSF proteins. All C-RASSF proteins have Ras association (RA) and Salvador/RASSF/Hippo (SARAH) domains. RASSF1A and NORE1 carry a C1 domain. RASSF6 has a PDZ-binding motif

pathway. Thereby, the presence of this domain distinguishes RASSF1–6 from RASSF7–10. Moreover, the RA domain resides in the N-terminus of RASSF7–10, whereas it is near the C-terminal region in RASSF1–6. Thus, RASSF7–10 are called N-RASSF proteins, whereas RASSF1–6 are known as C-RASSF proteins. Even though "C" generally denotes the C-terminus, it also indicates "classical." Numerous reports have demonstrated that *C-RASSF*s are suppressed in human cancers and that the suppression of each *C-RASSF* is correlated with tumor progression [4]. Based on these reports, *C-RASSF*s, excluding *RASSF1C*, are regarded as tumor suppressors. Various underlying mechanisms have been proposed for *C-RASSF*-mediated tumor suppression. RASSF1 and RASSF5 are well researched, whereas other C-RASSF proteins have been less thoroughly investigated. Nevertheless, it has been noted that C-RASSF proteins share common mechanisms of tumor suppression. Even though some mechanisms depend on other tumor suppressors such as p53, pRb, and the Hippo pathway, C-RASSF proteins also utilize unique mechanisms for suppressing tumors. These findings highlight the importance of *C-RASSF*s as tumor suppressors in cancers, especially with the dysregulation of p53, pRb, and the Hippo pathway. Moreover, accumulating evidence supports that C-RASSF proteins play roles other than in tumor suppression. In this review, we attempt to summarize the current knowledge of C-RASSF (Table 1).

Summary of the Hippo pathway

C-RASSF proteins cross-talk with the Hippo pathway. As this cross-talk is one of the important properties of C-RASSF proteins, we will briefly summarize the Hippo pathway (Fig. 2). For details, readers are requested to refer

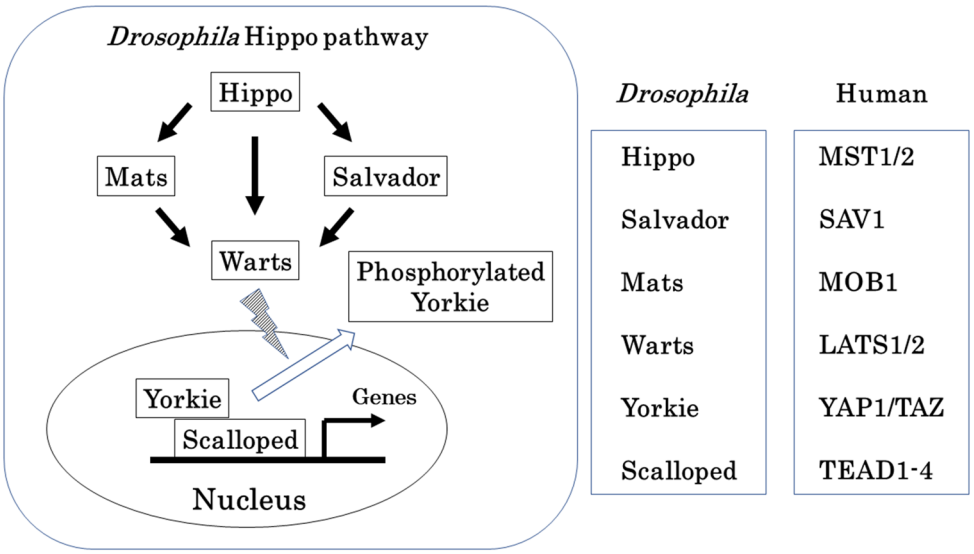

Fig. 2 Core architecture of *Drosophila* and the components of the mammalian Hippo pathway. The core components of the *Drosophila* Hippo pathway are depicted (left). Unphosphorylated Yorkie interacts with Scalloped in the nucleus. Hippo together with Mats and Salvador activates Warts. Activated Warts phosphorylates Yorkie. Phosphorylated Yorkie is trapped in the cytoplasm, where it undergoes degradation. Mammalian homologs are listed (right)

Table 1 Summary of the properties of C-RASSF proteins

Gene name	Roles in Ras signaling	Roles in the Hippo pathway	Roles in the regulation of p53	Unique characters
RASSF1A [31]	RASSF1A mediates RAS-induced apoptosis through BAX activation KRAS enhances RASSF1A-mediated stabilization of microtubules RASSF1A switches RAF1 from MST and increases MEK signaling	RASSF1A activates the Hippo pathway through the stabilization of MST, the prevention of the dephosphorylation of MST, and the release of MST from the inhibition by RAF1	RASSF1A enhances p53 expression through the disruption of the complex of USP7, DAAX, and MDM2	RASSF1A is associated with microtubules
RASSF1C [31]	No report	No report	No report	RASSF1C is possibly oncogene
RASSF2 [103]	KRAS enhances the interaction between RASSF2 and PAR-4 Proteomics analysis revealed molecules that interact with RASSF2 in the KRAS-dependent manner	RASSF2 activates MST	No report	RASSF2 is required for the normal development of bone and hematopoiesis
RASSF3 [114]	No report	No report	RASSF3 enhances p53 expression through blocking MDM2-mediated degradation	
RASSF4 [121]	KRAS augments RASSF4-mediated apoptosis	RASSF4 inhibits MST1 in alveolar rhabdomyosarcoma and activates YAP1	No report	RASSF4 is involved in phosphoinositide metabolisms and Ca^{2+} signaling RASSF4 may be oncogenic
NORE1A/RASSF5 isoform A [8]	NORE1A mediates KRAS-induced apoptosis via MST1 NORE1A induces the degradation of β-catenin and MDM2 and stabilizes HIPK2 in response to HRAS NORE1A inhibits the dephosphorylation of pRb in response to KRAS and HRAS	NORE1A blocks the homodimerization and activation of MST, but fails to inhibit the activated MST	NORE1A upregulates p53-mediated pro-apoptotic gene transcription through the suppression of MDM2 and the stabilization of HIPK2	The affinity for RAS proteins is the highest among C-RASSF proteins
NORE1B/RAPL/RASSF5 isoform C [8, 15]	No report for RAS but in lymphocytes, RAP1, which has the same effector sequence as RAS, regulates the traffic of integrin via NORE1B	No report	No report	
RASSF6 [125, 126, 131]	The interaction with MDM2 is strengthened by KRAS	RASSF6 suppresses MST activity and is prevented by MST from inducing apoptosis	RASSF6 enhances p53 through blocking MDM2-mediated degradation	RASSF6 binds to membrane-associated proteins containing PDZ domains
dRASSF (*D. melanogaster*) [141]	Loss-of-function of *dRASSF* recovers the cell growth in mutants with loss-of-function of *Ras1*, suggesting that dRASSF antagonizes Ras1	dRASSF suppresses the cell overgrowth in mutants with the kinase-negative Hippo dRASSF suppresses Hippo through dephosphorylation	No report	

Table 1 (continued)

Gene name	Roles in Ras signaling	Roles in the Hippo pathway	Roles in the regulation of p53	Unique characters
RSF-1 (*C. elegans*) [144]	Loss-of-function of *rsf-1* suppresses the multivulva phenotype of active mutants of *let-60*, suggesting that RSF-1 works downstream of Let-60	No report	No report	

Our knowledge of C-RASSF proteins besides RASSF1A and NORE1 is still significantly limited. Therefore, although many characters are reported only for RASSF1A or NORE1, it is not yet concluded which ones are unique for RASSF1A or NORE1

to other reviews [5–7]. Genetic studies of *Drosophila melanogaster* revealed several mutants exhibiting cell overgrowth. One causative gene was named *hpo,* because the phenotype was reminiscent of the hippopotamus. Mutations of other three genes (*sav*, *mats,* and *wts*) resulted in the same phenotypes. *hpo* and *wts* encode the serine/threonine protein kinases Hippo and Warts, respectively. Hippo phosphorylates and activates Warts. The proteins encoded by *sav* and *mats*, namely Salvador and Mats, respectively, interact with Hippo and Warts and facilitate the Hippo-mediated activation of Warts. Yorkie, a protein that was identified as a Warts-interacting protein, cooperates with the transcription factor Scalloped and upregulates cell cycle-promoting and anti-apoptotic genes. However, activated Warts phosphorylates Yorkie, and subsequently, phosphorylated Yorkie is segregated in the cytoplasm and degraded. That is, the kinase cassette formed by Hippo, Salvador, Mats, and Warts negatively regulates Yorkie and Scalloped. Loss-of-function mutations of *hpo*, *sav*, *mats,* and *wts* lead to Yorkie and Scalloped hyperactivation and result in cell overproliferation and tissue overgrowth. The pathway composed of these genes was named the Hippo pathway. Humans have homologs of these genes as follows: mammalian Ste20-like kinase (MST) 1 and 2 (the human genes are *STK4* and *STK3*, respectively) for Hippo; Sav1 (also called WW45) for Salvador; MOB1A and MOB1B for Mats; large tumor suppressor kinase (LATS) 1 and 2 for Warts; yes-associated protein 1 (YAP1) and transcriptional coactivator with PDZ-binding motif (TAZ) (also called WW domain-containing transcription regulator protein 1) for Yorkie; and TEA domain transcription factor (TEAD) 1–4 for Scalloped. Subsequent studies have continuously identified new components of the Hippo pathway. The entire picture of the Hippo pathway is complicated. It is currently obvious that YAP1/TAZ are regulated by other molecules than MST1/2 and LATS1/2. Regardless, the MST-LATS-YAP1/TAZ-TEAD axis is the core of the mammalian Hippo pathway, which is dubbed the canonical Hippo pathway.

Human RASSF proteins

NORE1/RASSF5

Discovery of NORE1/RASSF5

As the first identified C-RASSF was mouse RASSF5 [8], with human RASSF5 reported later [9], we will start this review with this protein. In the pioneering study, the researchers screened for a protein that bound the active form of HRAS, obtaining mouse RASSF5, and named it novel Ras effector (NORE) 1 [8]. It is unsurprising that only RASSF5 was detected in that screening, as the affinity

of RASSF5 for Ras proteins is high [10, 11]. The interaction between RASSF5 and Ras proteins is easily detected in vitro, whereas the detection of the interaction between Ras proteins and other C-RASSF proteins depends on the experimental conditions. Due to the historical background, *RASSF5* is frequently described as *NORE1*. We also use NORE1 in this review. There are two major isoforms with different N-terminal sequences (*NORE1A* and *NORE1B,* which correspond to *RASSF5* isoforms A and C, respectively). *NORE1A* is downregulated by hypermethylation in various cancers [4]. *NORE1B* is also suppressed in certain cancers [12]. DNA hypermethylation is not the only cause of this suppression. In oral cancer, miR-214 suppresses *NORE1* [13]. NORE1 is also suppressed at the protein level by the E3 ligase ITCH [14]. NORE1B has been studied as a Rap1-binding protein in the field of immunology and described as RAPL [15]. RAPL determines the spatial localization of integrin subunit 2 and mediates Rap1-triggered integrin activation in T lymphocytes. RAPL regulates the localization of CDKN1B (P27KIP1), and its deficiency causes lymphoproliferative disorders [16]. These findings support that both NORE1A and NORE1B are tumor suppressors.

NORE1 as a target of Ras proteins

NORE1 binds Ras proteins (KRAS, HRAS, MRAS, and RRAS) and it is considered a typical target of RAS [8, 17]. The active form of Ras drives NORE1 to induce apoptosis and senescence [18, 19]. The SARAH domain of NORE1 (NORE1-SARAH) interacts with the SARAH domains of MST kinases (MST-SARAH) [10, 18, 20]. NORE1 attenuates the autophosphorylation at threonine 183 of MST1, which is essential for MST1 activity, and inhibits MST1 activation [20]. However, when KRAS G12V is coexpressed, MST1 is recruited via NORE1 to the plasma membrane and activated [20]. In this manner, KRAS induces apoptosis via NORE1-MST1. On the other hand, HRAS prompts NORE1 to bind to the $SCF^{\beta\text{-}TRCP}$–ubiquitin ligase complex and induces the degradation of β-catenin and MDM2 [21, 22]. HRAS also induces NORE1 to bind and stabilize homeodomain-interacting protein kinase 2 (HIPK2) [19]. HIPK2 phosphorylates p53 at Serine 46, induces p53 acetylation, and eventually upregulates pro-apoptotic genes. HRAS and KRAS trigger the formation of a complex including NORE1 and protein phosphatase 1A (PP1A), allowing NORE1 to stabilize and bridge PP1A to pRb [23]. Consequently, pRb remains in its dephosphorylated active form and promotes cellular senescence. These findings support that NORE1 is controlled by Ras signaling and that it is a target of Ras proteins.

Interaction between NORE1 and MST kinases

MST kinases are mammalian homologs of yeast Ste20 kinases and core kinases of the Hippo pathway [24]. The interaction between NORE1 and MST kinases has been extensively studied [25–27]. The affinity of the homodimerization of MST-SARAH is weaker than that of heterodimerization between MST-SARAH and NORE1-SARAH. Thus, NORE1-SARAH blocks the homodimerization of MST-SARAH and inhibits the autoactivation of MST kinases, which is a prerequisite for MST kinase activity. Consistently, NORE1-SARAH fails to inhibit MST kinases once MST kinases are autophosphorylated and activated [28]. Moreover, both MST-SARAH and the N-terminal kinase domain (MST-N) bind NORE1-SARAH, and thus, NORE1-SARAH is sandwiched between MST-N and MST-SARAH [27, 29]. Moreover, the RA domain of NORE1 (NORE1-RA) binds to the region between MST-N and MST-SARAH, which is named the regulatory region (MST-RR). These findings indicate that NORE1 and MST kinases interact with each other at multiple sites. Hence, to understand the interaction between NORE1 and MST kinases, research using the whole molecules is important. Intriguingly, NORE1-SARAH enhances the phosphorylation of histone H2B by MST1 but attenuates the phosphorylation of FoxO [29]. This result implies that the effect of NORE1 on MST1 depends on the substrate. Therefore, to discuss the effect of NORE1 in the context of the Hippo pathway, it is essential to use Hippo pathway-related substrates such as MOB1 and LATS kinases.

By what mechanism does Ras signaling modulate the interaction between NORE1 and MST kinases? An experiment using mouse NORE1 revealed that NORE1-RA intramolecularly binds to the C1 domain (NORE1-C1), but that RAS releases NORE1-C1 from NORE1-RA [30]. It is presumed that Ras signaling triggers a conformational change of NORE1 and modulates the interaction between NORE1 and MST kinases.

RASSF1

Discovery of RASSF1

The human chromosome 3p21.3 frequently exhibits loss of heterozygosity in human cancers, which implies that a tumor suppressor is encoded in this region. Research to identify xeroderma pigmentosum A (XPA)-interacting proteins revealed a gene homologous to *NORE1* in this region that was reported as *RASSF1* [31]. In the first paper, three splicing variants were described, one of which was suppressed in human cancers through methylation of the CpG island promoter. This variant is the well-known tumor suppressor *RASSF1A*. Thereafter, numerous papers have reported that

RASSF1A suppression is associated with tumor progression and poor prognosis in human cancers. Several splicing variants of *RASSF1* are registered in the database of the National Center for Biotechnology Information, but most studies have focused on *RASSF1A* and *RASSF1C*.

RASSF1A stabilizes microtubules

Versatile mechanisms contribute to RASSF1A-mediated tumor suppression. Among them, microtubule stabilization is one of the most prominent mechanisms and one that is unique to RASSF1A. RASSF1A stabilizes microtubules via microtubule-associated proteins such as MAP1B and MAP1S (C19ORF5) [32–35]. The stabilization of microtubules by RASSF1A also depends on RAN [36]. RAN regulates nuclear transport, but it is also involved in the assembly of mitotic spindles. RASSF1A induces phosphorylation in the nuclear localization signal of regulator of chromosome condensation 1 (RCC1), the GDP/GTP exchanger of RAN, leading to RCC1 accumulation in the cytoplasm. As a result, RASSF1A increases the level of the GTP-bound form of RAN and stabilizes microtubules via RAN. Other proposed mechanisms underlying the stabilization of microtubules are the inhibition of histone deacetylase 6-mediated deacetylation of α-tubulin and the recruitment of protein arginine *N*-methyltransferase 5 to microtubules [37, 38]. An analysis of upregulated genes in the *Rassf1a*-deleted mouse liver revealed two categories of genes [39]. One group of genes is involved in microtubule polymerization, which underscores the importance of microtubule stabilization in the function of RASSF1A.

RASSF1A activates the Hippo pathway

The interaction between RASSF1A and MST kinases has long been recognized [10, 20]. Later, proteomic studies further identified RASSF1A as a component of the Hippo pathway [40]. The SARAH domain of RASSF1A (RASSF1-SARAH) binds MST1-SARAH similarly as NORE1-SARAH albeit with a slight difference [29]. Whether NORE1 activates or inhibits MST kinases depends on the context [28]. By contrast, RASSF1A activates MST kinases in vivo [41]. Several modes of activation have been proposed. RASSF1A stabilizes MST kinases [41]. RASSF1A prevents the dephosphorylation of MST kinases and maintains the active phosphorylated forms [42]. RASSF1A releases MST2 from inhibition by RAF1 [43]. Thus, RASSF1A activates MST kinases and drives the Hippo pathway. Earlier research revealed the role of RASSF1A in FAS-induced apoptosis [43]. FAS activates MST2 via RASSF1A and MST2 in turn activates LATS1. RASSF1A releases YAP1 from LATS1, induces the accumulation of YAP1 and p73 in the nucleus, and promotes the YAP1-p73–mediated transcription of pro-apoptotic genes. In this context, YAP1 is regarded as a tumor suppressor that cooperates with p73. A study using *Sleeping Beauty transposase* in *Rassf1a*-null mice demonstrated that YAP1 shifts from p73 to TEAD and RUNX2 in the *Rassf1a*-negative background and that additional *Runx2* depletion further enhances YAP1-TEAD complex formation [44]. Moreover, RASSF1A inhibits the TGF-β-induced interaction between YAP1 and SMAD2 [45]. Although RASSF1A is degraded by ITCH in response to TGF-β, the remaining RASSF1A restricts the nuclear translocation of SMAD2 and promotes cooperation between YAP1 and p73. These findings may explain the mechanism by which YAP1 selects a binding partner among various transcription factors and behaves as a tumor suppressor or tumor promoter in a context-dependent manner. Another paper reported that under RASSF1A overexpression, YAP1 is phosphorylated by LATS kinases [46]. Consequently, *AREG*, which is a target of TEAD that encodes a member of the epidermal growth factor family, is suppressed. In all of these scenarios, RASSF1A drives the Hippo pathway as an upstream regulator and causes apoptosis irrespective of whether YAP1 behaves as an oncogene or tumor suppressor. However, in the *Rassf1a*-depleted mouse liver, the expression levels of total YAP1 and phosphorylated YAP1 are not significantly changed [39]. This finding suggests that RASSF1A functions as a tumor suppressor through a different mechanism from the canonical Hippo pathway. For instance, RASSF1A interacts with SAV1 independently of MST kinases and activates p73-driven gene transcription [47]. A recent paper reported that RHEB, the activator of mTOR kinase, interacts with RASSF1A and inactivates YAP1 through MST and LATS kinases [48]. Conversely, RASSF1A blocks REHB-mediated autophagy. Consequently, RASSF1A suppresses RHEB-mediated anchorage-independent cell growth of tumor cells.

RASSF1A as a target of Ras proteins

Active KRAS induces apoptosis through RASSF1A [49]. KRAS also enhances RASSF1A-mediated stabilization of microtubules [34]. These findings support that RASSF1A is a target of Ras protein. However, RASSF1A has lower affinity for Ras proteins than NORE1 [10]. It is argued that RASSF1A indirectly binds Ras proteins via NORE1 [50]. The connector enhancer of kinase suppressor Ras 1 (CNKR1), which is reported to interact with RASSF1A and induce apoptosis via MST kinases, is also a candidate that links Ras proteins to RASSF1A [51]. Ras signaling may regulate RASSF1A via NORE1 or CNKR1. In addition, RASSF1A dissociates the complex of RAF1 and MST2, decreases the inhibitory phosphorylation of RAF1, and increases MEK activities. This observation suggests that RASSF1A functions as a modifier of Ras signaling [52].

The relationship of RASSF1A with p53 and pRb

p53 and pRb are apparently involved in the tumor suppressor function of RASSF1A. However, unlike NORE1, there is no clear evidence that RASSF1A transduces Ras signaling to p53 and pRb. Ubiquitin-specific protease 7, DAAX, and MDM2 form a complex and enhance p53 degradation [53]. Upon DNA damage, RASSF1A disrupts the complex and enhances p53 expression [54]. However, the role of RASSF1A does not depend solely on p53. *Rassf1a*-null mice exhibit tumor susceptibility [55, 56]. Importantly, *Rassf1a* deletion enhances tumor development, generates more aneuploid cells, and shortens survival in *p53*-null mice. Thus, RASSF1A functions as a tumor suppressor even in the p53-negative background. Through what mechanism does RASSF1A suppress tumor independently of p53? To address this question, we are focusing on pRb, another major tumor suppressor. RASSF1A restricts G1 exit in H1299 cells expressing pRb, but when E7 papillomavirus protein or CCNA2 (cyclin A2) is overexpressed (the former inhibits the interaction between pRb and E2F, and the later directly activates CDK2 and bypasses the regulation by pRb), RASSF1A-induced arrest is canceled [57]. These findings suggest that pRb is implicated in RASSF1A-mediated G1/S arrest.

RASSF1A regulates the cell cycle

In addition to stabilizing microtubules, RASSF1A regulates cell cycle progression by increasing the expression of cyclin-dependent kinase inhibitors. RASSF1A upregulates p53 and thereby enhances *CDKN1A*. However, RASSF1A upregulates *CDKN1A* even in A549 cells expressing the HPV16 E6 protein, which destabilizes p53, and in H1299 cells lacking p53 [58]. Thus, RASSF1A can induce *CDKN1A* independently of p53. It is proposed that RASSF1A negatively regulates AKT, which suppresses CDKN1A [58]. Another group reported that RASSF1A suppresses HRAS-induced c-Jun N-terminal kinase (JNK) activation and blocks JNK-induced downregulation of *CDKN1B* [59]. Furthermore, RASSF1A decreases the expression of cyclin-dependent kinases and cyclins. RASSF1A reduces CDK4 expression by inducing miR-711, which targets *CDK4* [60]. RASSF1A reduces *CCNA2* and *CCND1* (cyclin D1) expression. RASSF1A binds E4F1 and inhibits its association with the promoter of *CCNA2* to induce its downregulation [61, 62]. RASSF1A affects the translation and stability of *CCND1* mRNA and blocks the accumulation of CCND1 [57, 63].

Moreover, RASSF1A regulates cell cycle progression through E3 ligase complexes. Although whether RASSF1A directly interacts with CDC20 is controversial, one report argued that RASSF1 induces mitotic arrest by inhibiting CDC20 and anaphase-promoting complex (APC/C) [64, 65]. MAP1S, which bridges RASS1A to microtubules, is believed to augment the interaction between RASSF1A and CDC2 [66]. Paradoxically, RASSF1A depletion impairs cell proliferation in certain cells. This phenomenon is explained by the aberrant activation of APC/C during G1/S transition [63]. RASSF1A blocks β-TRCP-mediated degradation of Emi1, the inhibitor of APC/C, and allows the accumulation of CCNA and CCNB, which is necessary for the G1/S transition. Thus, RASSF1A is likely to positively and negatively regulate the cell cycle.

RASSF1A is required for DNA repair

As RASSF1A regulates the cell cycle and checkpoints [57, 64], it is unsurprising that RASS1A deletion impairs DNA repair. In this section, we present specific findings that directly link RASSF1A to DNA repair. RASSF1A reduces the CDK2-mediated phosphorylation of BRCA2, an essential component of the error-free DNA repair machinery of DNA double-strand breaks, through MST2 and LATS2, and blocks the disassembly of the recombinase RAD51 from BRCA2 to protect genome stability [67]. RASSF1A interacts with XPA and modulates the interaction between XPA and replication protein A [31, 68]. In this manner, RASSF1A is involved in homologous recombination and nucleotide excision repair.

RASSF1A regulates apoptosis

In addition to the Hippo pathway and p53, RASSF1A regulates apoptosis through modulator of apoptosis 1 (MOAP1) [69]. Tumor necrosis factor (TNF)-α and TNF-like apoptosis-inducing ligand (TRAIL) induce the recruitment of RASSF1A and MOAP1 to the receptor complexes [70]. RASSF1A binds to 14-3-3 in the basal state, but when stimulated by TNF-α and TRAIL, RASSF1A is dissociated from 14-3-3 and binds to MOAP1. Then, RASSF1A releases MOAP1 from intramolecular autoinhibition and triggers its association with BAX, resulting in the insertion of BAX into the mitochondrial membrane and cytochrome c release. RASSF1A also transduces signaling from FAS and the TNF-α receptor to MST1, MOB1, and NDR (STK38 and STK38L) kinases and induces apoptosis via NDR kinases [71].

RASSF1A is regulated by phosphorylation

RASSF1A has several phosphorylation sites. As expected, phosphorylation modulates the interaction between RASSF1A and its binding partners. Phosphorylation at serines 175, 178, and 179 by glycogen synthase kinase 3β is necessary for the binding of RASSF1A to 14-3-3 [72]. Phosphorylation at threonine 202 and serine 203 by MST1

is involved in the activation of NDR kinases in response to TNF-α [71]. Phosphorylation at serine 184 by checkpoint kinase 1 disrupts the association of RASSF1A with microtubules [73, 74]. Among numerous phosphorylation sites, serines 131 and 203 may be most important. Serine 131 is phosphorylated by ataxia telangiectasia mutated (ATM) in response to DNA damage [75]. Phosphorylation promotes the dimerization of RASSF1A and the association of MST2 and LATS1, stabilizes YAP1 and p73, and subsequently enhances *CDKN1A* expression. The polymorphism that converts alanine 133 to serine disrupts α helix-containing ATM recognition sites and compromises p53/p73 responses. Accordingly, RASSF1A A133S is associated with poor prognosis in patients with sarcoma and early onset breast cancer in BRCA1/2 mutation carriers [76, 77]. Serine 203 is phosphorylated by several kinases. RASSF1A activates Aurora A, and it is reciprocally phosphorylated at serine 203. Phosphorylation by Aurora A triggers the dissociation of RASSF1A from both microtubules and CDC20 [73, 78]. Serine 203 is also phosphorylated by Aurora B, the isoform of Aurora A, but in the late mitosis phase [79]. Subsequently, RASSF1A binds syntaxin 16, a component of t-SNARE, at the midzone/midbody. In this manner, RASSF1A is involved in membrane trafficking during cytokinesis. CDK4, protein kinase A (PKA), and protein kinase C (PKC) phosphorylate serine 203 [80–82]. Phosphorylation by CDK4 induces RASSF1A degradation through an interaction with Skp2, the subunit of the Skp1-Cul1-F-box ubiquitin ligase complex, and promotes G1/S progression [80]. PKC phosphorylates serine 197 in addition to serine 203 [82]. PKC-mediated phosphorylation at these sites likely prevents the regulation by RASSF1A of microtubules. These two findings imply that phosphorylation at serine 203 negatively regulates the tumor suppressor function of RASSF1A. However, inhibition of PKA-mediated phosphorylation compromises RASSF1A-mediated apoptosis and the upregulation of *CDKN2A* and *BAX*, meaning that PKA-mediated phosphorylation at serine 203 promotes the tumor suppressor function of RASSF1A [81]. It is difficult to elucidate the mechanism by which phosphorylation at the same site leads to different cellular consequences. We may need to consider the localization of RASSF1A and the combination of various phosphorylations. Temporal and spatial analyses of the phosphorylation of RASSF1A are essential for clarifying the mechanism by which RASSF1A orchestrates various cellular events in response to the pattern of phosphorylation.

The other mechanisms underlying the tumor suppressor roles of RASSF1A

RASSF1A regulates Rho signaling. The C-terminal region of RASSF1A binds active RHOA, whereas the N-terminal region interacts with Smad ubiquitin regulatory factor 1 and induces RHOA degradation [83]. Conversely, RASSF1A stimulates the cofilin/PP2A-mediated dephosphorylation of the guanine nucleotide exchange factor GEF-H1 and activates RHOB [84]. RHOB suppresses nuclear YAP1 and plays an anti-metastatic role. RASSF1A reduces estrogen receptor α expression through AKT and inhibits breast tumor growth [85]. Rac1 is activated in Rassf1-depleted mouse embryonic fibroblasts, suggesting that RASSF1A regulates Rac signaling [86]. MAP1S, which was previously described as a microtubule-associated protein, is involved in the biogenesis and degradation of autophagosomes [87]. As deregulation of autophagy is associated with tumorigenesis, it will be necessary to study the mechanism by which RASSF1A affects autophagy. RASSF1A blocks the $SCF^{\beta\text{-}RTRCP}$-mediated degradation of repressor element 1 silencing transcription factor and, consequently, downregulates the oncogenic factor miR-21, which targets various tumor suppressor genes such as *PTEN* [88]. All these properties may also contribute to the tumor suppressor function of RASSF1A.

RASSF1A may be implicated in non-cancer diseases

RASSF1A restricts Toll-like receptor-stimulated NFκB signaling [89]. RASSF1A interacts with and inhibits Tank binding kinase 1, the activator of NFκB signaling [39]. Correspondingly, *Rassf1a*-null mice displayed enhanced inflammatory reaction in a dextran sulfate sodium-induced colitis model [89]. RASSF1A and MST1 antagonize TNF-α signaling in cardiac myocytes and fibroblasts and block fibrosis [90, 91]. RASSF1A interacts with ATP2B4 (plasma membrane calmodulin-dependent calcium ATPase) in the heart [92]. RASSF1A depletion causes cardiac hypertrophy [90, 91]. Interestingly, the expression of genes related to the circadian clock is altered in the *Rassf1a*-deleted mouse liver [39]. RASSF1A may have additional roles other than tumor suppression. Investigation of the implication of RASSF1A in non-cancerous diseases will have clinical significance.

RASSF1C is an oncogene

Initially, RASSF1C was considered a tumor suppressor similarly as RASSF1A. It was reported that RASSF1C activates SAPK/JNK signaling, triggers senescence and apoptosis, and plays a tumor suppressor role in prostate cancer and renal carcinoma cells and that RASSF1C increases the sensitivity to CDDP in ovarian cancer cells [93–95]. Nevertheless, accumulating evidence has overturned this belief. RASSF1C is upregulated in breast, lung, esophageal, and pancreatic endocrine tumors [96, 97]. CpG islands are not hypermethylated in the promoter of RASSF1C. RASSF1C inhibits the β-TRCP-mediated degradation of β-catenin [98]. RASSF1C enhances the expression of genes implicated in

cancer development, such as *PIWIL1* [97, 99]. Furthermore, RASSF1A regulates lung cell transformation and tumorigenesis through modulating the expression of PIWI-interacting RNAs [100]. In cells with methylated *RASSF1A*, RASSF1C expression is alternatively enhanced, and the protein interacts with SRC and YES1 and increases the tyrosine phosphorylation of YAP1 [101]. Furthermore, RASSF1C promotes the SRC-dependent phosphorylation of E-cadherin and destabilizes cell junctions. As a result, RASSF1C increases nuclear β-catenin levels. Moreover, RASSF1C induces MYC. These findings strongly support that RASSF1C is an oncogene. It is known that RASSF1A and RASSF1C play opposite roles in cell proliferation and apoptosis in breast and lung cancer cells [102]. RASSF1C expression is high in breast and lung tumors, whereas RASSF1A expression is low. The expression ratio of RASSF1A and RASSF1C may be an important determinant of tumor properties.

RASSF2

Identification of RASSF2 as a tumor suppressor

RASSF2 was reported as the third member of the RASSF protein family [103]. Enforced expression of RASSF2 causes cell cycle arrest and apoptosis. Deletion of *RASSF2* enhances tumorigenicity and drug resistance in lung cancer cells. *RASSF2* silencing via hypermethylation has been reported in various human cancers [4, 104, 105]. Cancer-associated fibroblasts produce miR-7, which suppresses *RASSF2* expression [106]. Enhancer of zeste homolog 2, which is overexpressed in cancers, downregulates *RASSF2* [107]. These data indicate that RASSF2 is a tumor suppressor.

The molecular mechanism underlying the tumor suppressor role of RASSF2

We do not yet fully understand the mechanism by which RASSF2 suppresses tumors. Several papers reported the inhibition of NFκB signaling, the activation of MST kinases and JNK, and the nuclear recruitment of prostate apoptosis response protein-4 (PAR-4) as the underlying mechanisms [108–111].

RASSF2 as a target of Ras proteins

Active KRAS enhances the interaction between RASSF2 and PAR-4 [110]. Furthermore, proteomics analysis revealed several molecules that bind RASSF2 in the KRAS-dependent manner [112]. These findings suggest that RASSF2 is a target of KRAS.

Other properties of RASSF2

Rassf2-null mice exhibit bone defects and hematopoietic abnormalities [108]. This phenotype indicates that RASSF2 physiologically plays a role other than tumor suppression. As each C-RASSF displays a distinct distribution in cells, regulation of the subcellular localization of C-RASSF is an important issue to study. In this light, the finding that the nuclear cytoplasmic shuttle of RASSF2 is regulated by extracellular regulated kinase 2 is interesting [113].

RASSF3

Our understanding of RASSF3 lags far behind that of other C-RASSFs. *RASSF3* was reported as a homolog of *RASSF1A* [114]. *Rassf3* causes resistance to mammary tumor development in *neu*-transgenic mice [115]. In humans, *RASSF3* downregulation is detected in non-small cell lung cancer, and it is correlated with disease progression [116]. We reported that RASSF3 regulates apoptosis and the cell cycle via p53 and contributes to tumor suppression [117]. These findings support that RASSF3 is a tumor suppressor.

RASSF4

Whether RASSF4 is a tumor suppressor is elusive. RASSF4 suppression is observed in non-small cell lung cancer, nasopharyngeal carcinoma, and multiple myeloma [118–120]. RASSF4 overexpression induces apoptosis and inhibits proliferation in HEK293 cells [121]. RASSF4 reduces β-catenin, MYC, and CCND1 expression in osteosarcoma cells [122]. These properties support that RASSF4 is a tumor suppressor. Unexpectedly, however, RASSF4 is upregulated in alveolar rhabdomyosarcoma (aRMS), in which it activates YAP1 and promotes tumorigenesis [123]. The researchers explained that RASSF4 inhibits MST1 and suppresses the phosphorylation of YAP1. As we will expound upon in the section "RASSF6", MST kinases and some, if not all, C-RASSF proteins inhibit each other under the basal condition. This mutual inhibition may be meaningful for avoiding excessive cell death. However, when cells are exposed to stresses such as DNA damage, MST kinases and C-RASSF are released from inhibition, after which they mediate apoptosis in a parallel manner. Suppose that the downstream tumor suppressive mechanism of C-RASSF proteins is impaired. In this situation, high expression of C-RASSF proteins may compromise the Hippo pathway and lead to tumorigenesis. If this scenario is correct, then the finding that RASSF4 activates YAP1 in aRMS is consistent with its original property as a tumor suppressor. However, a recent study revealed another possibility. RASSF4 interacts with the GDP-bound form of adenosine diphosphate ribosylation factor 6 (ARF6) and activates type I phosphatidylinositol phosphate kinase

to enhance phosphatidylinositol 4,5-biphosphate (PI(4,5)P2) levels [124]. In response to this, the endoplasmic reticulum (ER) Ca^{2+} sensor stromal interaction molecule 1 is accumulated at ER–plasma membrane junctions, and store-operated Ca^{2+} entry is triggered. RASSF4 may contribute to tumorigenesis through phosphoinositide metabolisms and Ca^{2+} signaling.

RASSF6

Identification of RASSF6

RASSF6 was initially identified as a gene encoded in the bronchiolitis susceptibility locus [125]. Later, RASSF6 was characterized as a C-RASSF protein based on its sequence homology [126]. RASSF6 suppression by DNA hypermethylation is frequently observed in various human cancers [127–129]. In gastric cancer, miR-181a-5p suppresses RASSF6 [130]. Thereby, RASSF6 is considered a typical tumor suppressor. We identified RASSF6 in yeast two-hybrid screening using membrane-associated guanylate kinase inverted 1 (MAGI1) as bait [131]. The MAGI family consists of three members, MAGI1, MAGI2, and MAGI3 [132–134]. MAGI proteins have multiple PDZ, two WW, and one guanylate kinase domain. RASSF6 binds to the PDZ domains of MAGI proteins through the C-terminal PDZ-binding motif, the sequence of which distinguishes RASSF6 from other C-RASSF proteins. RASSF6 interacts with DLG1, which also has PDZ domains [135]. MAGI proteins and DLG1 are components of polarized epithelial junctions. MAGI2 is known as a tumor suppressor. Therefore, the interaction between RASSF6 and these proteins is intriguing, and its physiological significance must be clarified.

The molecular mechanism underlying the tumor suppressor role of RASSF6

RASSF6 causes apoptosis via caspase-dependent and caspase-independent mechanisms [131]. Conversely, RASSF6 depletion attenuates apoptosis caused by TNF-α, okadaic acid, high osmolarity, and ultraviolet radiation [131, 136–138]. These findings indicate that RASSF6 mediates apoptosis under various conditions. RASSF6 activates BAX via MOAP1 and promotes the release of cytochrome c, apoptosis-inducing factor, and endonuclease G from mitochondria [126, 131, 136]. Mechanistically, RASSF6 interacts with MDM2 and blocks the MDM2-mediated degradation of p53 [137]. KRAS augments the interaction between RASSF6 and MDM2 [139]. The RA domain (RASSF6-RA) binds to the C-terminal RING domain of MDM2. The SARAH domain (RASSF6-SARAH) intramolecularly binds to RASSF6-RA and inhibits the interaction between RASSF6-RA and MDM2. KRAS releases RASSF6-RA from this inhibition and shifts it toward binding to MDM2. In this manner, RASSF6 mediates Ras-induced p53-dependent apoptosis, supporting that RASSF6 is a target of Ras signaling. RASSF6 inhibits NFκB and MAPK signaling and promotes CDKN1A accumulation via JNK [126, 140]. These properties also contribute to tumor suppression.

RASSF6 cooperates with the Hippo pathway

RASSF6 is the highly pro-apoptotic protein. When RASSF6 is exogenously expressed, most cells do not survive for a long period, but when MST kinases are coexpressed, RASSF6-induced apoptosis is remarkably suppressed [136]. Contrarily, RASSF6 inhibits MST kinases. This mutual inhibition is mediated by the SARAH domains. Coexpression of full-length RASSF6 blocks the autophosphorylation of MST kinases in vivo and inhibits the phosphorylation of MOB1 in vitro. However, okadaic acid treatment dissociates RASSF6 and MST kinases from each other. Consequently, RASSF6-mediated apoptosis is triggered, and the Hippo pathway is simultaneously activated. Based on this finding, we speculate that RASSF6 and the Hippo pathway cooperatively function as tumor suppressors. In this respect, RASSF6 is different from RASSF1A in that it drives the Hippo pathway to suppress tumors.

C-RASSF proteins of non-mammalian organisms

C-RASSF of *D. melanogaster*

Drosophila melanogaster expresses one C-RASSF named dRASSF. *Drosophila* cells with *Ras1* loss-of-function mutations exhibit growth defects, but additional mutation of *dRASSF* rescues the phenotype [141]. This implies that dRASSF antagonizes Ras1 and participates in Ras signaling. Likewise, the relationship with the Hippo pathway is conserved. dRASSF physically interacts with Hippo via the SARAH domain. dRASSF suppresses the overgrowth phenotype of the kinase-negative Hippo mutant but has no effect on the SARAH domain-lacking Hippo mutant [141]. This observation is comprehensible if dRASSF suppresses Hippo similarly as RASSF6 inhibits MST kinases via the SARAH domains. Proteomic analysis revealed that dRASSF interacts with the *Drosophila* striatin-interacting phosphatase and kinase (dSTRIPAK) complex and represses Hippo through dephosphorylation, thus functioning in contrast to RASSF1A, which that activates MST kinases through the inhibition of dephosphorylation [142]. Mutation of lethal (2) giant larvae, a regulator of apical-basal cell polarity, results in the mislocalization of Hippo, dRASSF, and dSTRIPAK [143]. This finding corroborates that these proteins form a

complex. Given that NORE1 promotes pRb dephosphorylation via PP1A and that RASSF1A blocks of MST kinase dephosphorylation by PP2A, the regulation of dephosphorylation may be one of common functions of C-RASSF proteins [23, 42].

C-RASSF of *Caenorhabditis elegans*

In the first paper that reported NORE1, the researchers identified *C. elegans T24F1.3* as a homolog of *NORE1* [8]. Later, we studied *T24F1.3* in *C. elegans*, characterized the mutant, and named the gene *rsf-1* [144]. RSF-1 interacts with CST-1/2, which are homologs of MST kinases. However, as CST-1/2 are not involved in the regulation of Wts, the homolog of LATS kinases, RSF-1 is irrelevant to the Hippo pathway, and the loss-of-function mutant of *rsf-1* does not exhibit a phenotype related to cell proliferation and apoptosis. Importantly, *rsf-1* mutation suppresses the multivulva phenotype of active mutants of *let-60*, the homolog of *RAS*. This observation suggests that RSF-1 is implicated in Ras signaling. Moreover, RSF-1 interacts with Rab-39, a small GTP-binding protein [145]. *rsf-1* silencing and *rab-39* mutation make worms more sensitive to oxidative stress. As RASSF1A binds RHOA and RASSF4 interacts with ARF6, we can assume that C-RASSF proteins interact with several GTP-binding proteins other than Ras proteins.

Perspective

The number of research papers concerning C-RASSF proteins has continuously grown, but the progress has not been equal for all C-RASSFs. For example, regulation by phosphorylation is well researched for RASSF1A but not for other C-RASSF proteins. Nonetheless, we currently know several mechanisms instrumental for the tumor suppressor functions of C-RASSF proteins. It is difficult to believe that all mechanisms work simultaneously. Moreover, it is unlikely that all C-RASSF proteins equally contribute to tumor suppression in all tissues. Research to determine the mechanism by which each C-RASSF functions in various tissues, cells, and subcellular compartments and under various conditions will be indispensable for clarifying the whole picture of C-RASSF. We also need to raise a very naïve question. Why do mammals have so many C-RASSF proteins? It is unquestionable that C-RASSF proteins can interact with each other when they are colocalized in cells [135]. MST1 and MST2 form a heterodimer with lower kinase activity than their homodimers [146]. Accordingly, we can hypothesize that heterodimers of C-RASSF proteins have different activities and that mammals require the fine-tuning of C-RASSF proteins to maintain tissue homeostasis. Research to dissect the relationships among C-RASSF proteins is important. From the clinical viewpoint, reactivation of *C-RASSF* expression via epigenetic reactivation is a reasonable strategy for cancer therapy. The simple idea is to inhibit DNA methylation. However, if we identify a certain mechanism that is pivotal for C-RASSF proteins to function as tumor suppressors, we can develop a surrogate method to compensate for the function of C-RASSF in cancer cells with *C-RASSF* downregulation. Such perspectives motivate us to study C-RASSF.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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