The RASSF1A tumor suppressor

Howard Donninger[1], Michele D. Vos[2] and Geoffrey J. Clark[1,*]

[1]Molecular Targets Group, Department of Medicine, J. G. Brown Cancer Center, University of Louisville, 119C Baxter Boulevard, 580 S. Preston Street, Louisville, KY 40202, USA
[2]Research Analysis and Evaluation Branch, NCI, Rockville, MD, USA
*Author for correspondence (e-mail: gjclar01@louisville.edu)

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Summary

RASSF1A (Ras association domain family 1 isoform A) is a recently discovered tumor suppressor whose inactivation is implicated in the development of many human cancers. Although it can be inactivated by gene deletion or point mutations, the most common contributor to loss or reduction of RASSF1A function is transcriptional silencing of the gene by inappropriate promoter methylation. This epigenetic mechanism can inactivate numerous tumor suppressors and is now recognized as a major contributor to the development of cancer.

RASSF1A lacks apparent enzymatic activity but contains a Ras association (RA) domain and is potentially an effector of the Ras oncoprotein. RASSF1A modulates multiple apoptotic and cell cycle checkpoint pathways. Current evidence supports the hypothesis that it serves as a scaffold for the assembly of multiple tumor suppressor complexes and may relay pro-apoptotic signaling by K-Ras.

Key words: Epigenetic, RASSF1A, Ras, Tumor suppressor

Introduction

For many years it was suspected that one or more tumor suppressors lurk in the 3p21.3 region of the human genome, because this area frequently suffers loss of heterozygosity (LOH) in lung cancer (Lerman and Minna, 2000). In 2000, Damman et al. serendipitously cloned a gene located in this region that they termed RASSF1 (Ras association domain family 1), because the protein contains a putative Ras association (RA) domain. One of the isoforms produced by the gene, RASSF1A, has properties compatible with a tumor suppressor function. Moreover, the gene appears to suffer frequent transcriptional inactivation in tumor cells due to aberrant promoter methylation (Burbee et al., 2001; Dammann et al., 2000). Simultaneously, a bioinformatics-based approach revealed a proapoptotic novel Ras-binding protein that inhibits tumor cell growth and is encoded by a gene localizing to 3p21.3. This protein turned out to be RASSF1C, a smaller isoform produced by the *RASSF1* gene (Vos et al., 2000). Thus, RASSF1 appeared to be one of the elusive tumor suppressors located at 3p21.3.

Subsequent work showed that specific point mutations compromise the ability of RASSF1A to inhibit tumor cell growth (Dreijerink et al., 2001; Kuzmin et al., 2002; Shivakumar et al., 2002). A small deluge of papers began, demonstrating frequent epigenetic inactivation of RASSF1A in a wide variety of tumors. Bearing in mind that RASSF1A can also suffer point mutations in up to 15% of primary tumors (Pan et al., 2005), RASSF1A is one of the most frequently inactivated proteins ever identified in human cancer.

RASSF1A lacks any obvious enzymatic activity but may serve as a scaffold for signaling complexes, key components of which have recently been identified (Fig. 1). Here, we discuss work that has implicated RASSF1A in the regulation of the cell cycle, apoptosis and genetic instability (Agathanggelou et al., 2005), and the molecular mechanisms involved.

RASSF1A is a tumor suppressor

Tumor suppressor genes are classically defined by Knudson's 'two-hit' hypothesis (Knudson, 1971), which states that inactivation of both alleles of a tumor suppressor gene is required for tumorigenesis. Loss of a *RASSF1A* allele is a frequent phenomenon in primary human cancer (Burbee et al., 2001; Pfeifer and Dammann, 2005). In a study of sporadic lung cancers, 76% of the tumors showing allelic imbalance at 3p21.3 (the *RASSF1A* locus) also showed *RASSF1A* promoter hypermethylation (Agathanggelou et al., 2001). Similar findings in non-small-cell lung cancer (NSCLC) (Tomizawa et al., 2002), bladder transitional carcinoma (Chan et al., 2003) and cervical squamous cell carcinoma (Yu et al., 2003) have been reported. Thus *RASSF1A* alleles can be inactivated by a combination of genetic and epigenetic mechanisms, and RASSF1A conforms to the Knudson two-hit model. Hypermethylation of both alleles of the *RASSF1A* promoter has been shown to cause loss of expression of the gene (Lusher et al., 2002). Moreover, although early studies reported infrequent mutation of RASSF1A, other studies have suggested that up to 15% of tumors may contain inactivating point mutations (Pan et al., 2005). Thus, the evidence that RASSF1A is inactivated in a high percentage of human tumors is strong (Table 1).

If the inactivation of RASSF1A contributes to the development of the transformed phenotype, then one might expect that re-introduction of RASSF1A into RASSF1A-negative cells would impair tumorigenicity. Indeed, this is the case. Re-expression of the gene in RASSF1A-negative cancer cells results in reduced colony formation in soft agar and reduced tumorigenicity in nude mice (Burbee et al., 2001; Dammann et al., 2000; Dreijerink et al., 2001; Kuzmin et al., 2002). Two groups have independently knocked out the RASSF1A gene in mice (Tommasi et al., 2005; van der Weyden et al., 2005). In each case the animals exhibit an

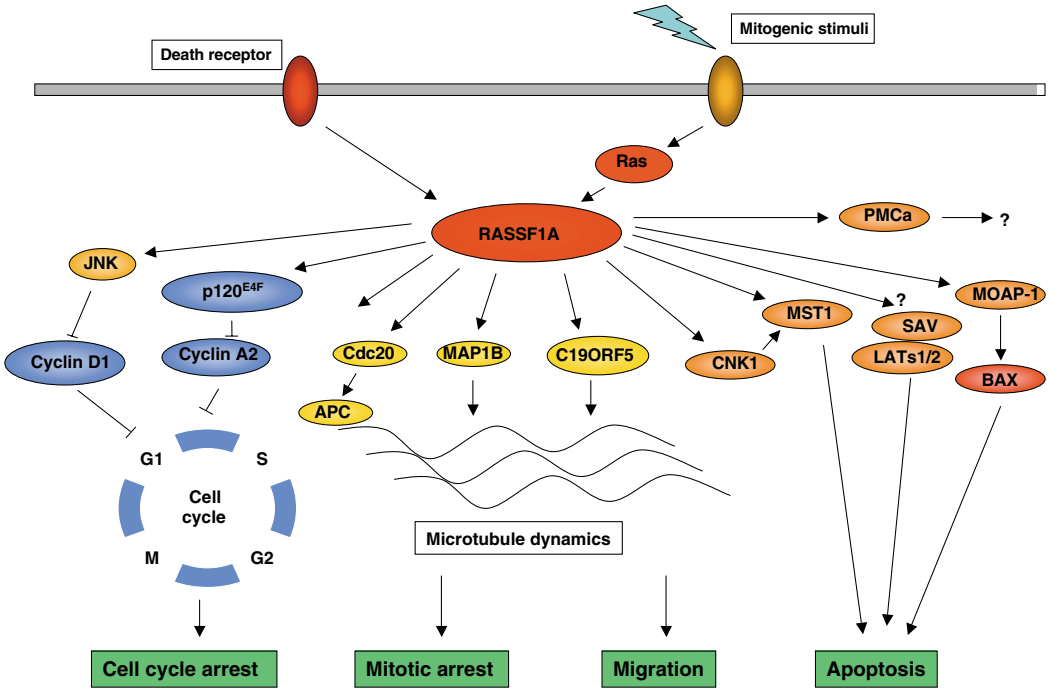

Fig. 1. Summary of some of the known partners and pathways of RASSF1A. RASSF1A binds to at least three microtubule-binding proteins (MAPs), complexes with microtubules and regulates mitosis, the cell cycle and apoptosis in response to mitogenic or apoptotic stimuli. Direct interaction between RASSF1A and microtubule-associated proteins localizes RASSF1A to the microtubules, stabilizing them and, thereby, regulating mitosis. Repression of cyclins A and D1 by RASSF1A results in cell cycle arrest and interactions with CNK1, MST1, Salvador and MOAP1 may allow RASSF1A to modulate apoptosis.

enhanced tendency to develop spontaneous tumors. Thus, RASSF1A is a tumor suppressor that is frequently impaired in human tumors.

RASSF1A is part of a family of potential tumor suppressors

RASSF1A is a member of a family of six related proteins, each of which exhibits multiple splice variants. With the exception of some minor splice variants, each protein contains an RA domain and a C-terminal SARAH protein-protein interaction motif. Each family member, with the exception of RASSF3, has now been implicated as a human tumor suppressor.

RASSF5 (Nore1a) is the best characterized member of the family after RASSF1A. RASSF5 was the first member of the family cloned and it was originally designated Nore1a for novel Ras effector 1 (Vavvas et al., 1998). RASSF5 binds activated Ras directly and is present in an endogenous complex with Ras in cells. RASSF5 is pro-apoptotic and kills cells in a Ras-dependent manner (Khokhlatchev et al., 2002; Vos et al., 2003a). It is frequently inactivated in human tumors by promoter methylation (Table 2). Moreover, it is linked to the development of a rare familial form of cancer (Chen et al., 2003), which confirms its role as a tumor suppressor in vivo. Its mechanisms of action remain largely unknown, although it may regulate the pro-apoptotic kinase MST1 (Praskova et al., 2004). A smaller splice version of RASSF5 has been identified and designated Nore1b or RAPL (Katagiri et al., 2003; Tommasi et al., 2002). This protein demonstrates more restricted expression than RASSF5 and can form an endogenous complex with the Ras-related protein Rap. It regulates lymphocyte adhesion and has also been implicated as a tumor suppressor (Katagiri et al., 2003; Macheiner et al., 2006).

RASSF2 is a pro-apoptotic Ras effector that is frequently downregulated in human tumors by promoter methylation, histone deacetylation and sometimes deletion (Akino et al., 2005; Endoh et al., 2005; Hesson et al., 2005; Lambros et al., 2005; Vos et al., 2003b). Inactivation of RASSF2 correlates with activation of Ras in tumor cells (Hesson et al., 2005). Reintroduction of RASSF2 into tumor cells impairs tumorigenesis and knocking down RASSF2 enhances tumorigenesis (Akino et al., 2005). Thus, RASSF2 also appears to be an epigenetically inactivated tumor suppressor. The effector pathways controlled by it remain unknown.

RASSF3 can also bind to Ras and inhibit cell growth (unpublished observations). However, it is the only family member whose RNA is not downregulated in tumors (Tommasi et al., 2002). Whether it is involved in tumor suppression thus remains unknown.

RASSF4 is frequently downregulated by promoter methylation in human tumor cells, binds to Ras and induces apoptosis. It localizes mostly to the cytosol but can be recruited to the plasma membrane by activated Ras (Eckfeld et al., 2004).

RASSF6 exhibits similar biological properties to its brethren and is often downregulated in primary human tumors (Allen et al., 2007). Intriguingly, the RASSF6 locus is implicated in susceptibility to bronchiolitis induced by respiratory syncytial virus (Hull et al., 2004). RASSF6 might therefore have a role in inflammation; indeed it can suppress the NFκB pathway (Allen et al., 2007).

Two further RA-domain-containing proteins have been identified and are now being described as RASSF8 (Falvella et al., 2006) and RASSF7. These were previously known as HOJ-1 and HRC1. Although these proteins can bind to Ras and inhibit cell growth (our unpublished observations), they do not display great homology with RASSF1-RASSF6 and do not contain SARAH motifs. This raises the issue of how to define a RASSF protein. RASSF proteins can heterodimerize with each other (Ortiz-Vega et al., 2002) and this might serve as a functional definition. Although we have found that RASSF2-RASSF6 readily heterodimerize with RASSF1A, the RASSF7

Table 1. Primary tumors containing *RASSF1A* promoter methylation

Tumor type	Frequency*	References
Lung: SCLC	88%	Grote et al., 2006
Lung: NSCLC	39% 28% 15%	Chen et al., 2006; Grote et al., 2006; Safar et al., 2005
Breast	95% 81%	Yeo et al., 2005; Shinozaki et al., 2005
Colorectal	20% 52%	Miranda et al., 2006; Oliveira et al., 2005
Prostate	99%	Jeronimo et al., 2004
Cervical Adenocarcinoma	45%	Cohen et al., 2003
Esophageal	34%	Wong et al., 2006
Gastric	44%	Oliveira et al., 2005
Renal	56-91%	Yoon et al., 2001; Dreijerink et al., 2001
Hepatocellular	75%	Katoh et al., 2006
Bladder	30-50%	Marsit et al., 2006
Pancreatic	63%	Liu et al., 2005a
Ovarian	26% 30%	Teodoridis et al., 2005; Makarla et al., 2005
Nasopharyngeal	68%	Tan et al., 2006
Leukemia	0% 15%	Johan et al., 2005; Harada et al., 2002
Neuroblastoma	83%	Lazcoz et al., 2006
Thyroid	71% 35%	Schagdarsurengin et al., 2006; Nakamura et al., 2005
Cholangiocarcinoma	67%	Tischoff et al., 2005
Ependymoma	36% 86%	Michalowski et al., 2006; Hamilton et al., 2005
Glioma	57% 54%	Hesson et al., 2004 Horiguchi et al., 2003
Hodgkin Lymphoma	65%	Murray et al., 2004
Medulloblastoma	79%	Lusher et al., 2002
Retinoblastoma	59%	Harada et al., 2002
Testicular Seminoma	40%	Honorio et al., 2003
Testicular Nonseminoma	83%	Honorio et al., 2003
Wilms tumor	54%	Wagner et al., 2002
Rhabdomyosarcoma	61%	Harada et al., 2002
Pheochromocytomas	22%	Astuti et al., 2001
Head and neck	15% 17%	Dong et al., 2003; Hogg et al., 2002
Melanoma	41%	Spugnardi et al., 2003

*Frequency of *RASSF1A* promoter hypermethylation in tumor type.

and RASSF8 proteins do not (our unpublished observation). Thus, RASSF7 and RASSF8 may be a separate sub-family distinct from the 'true' RASSF proteins.

RASSF1 produces multiple isoforms

The *RASSF1* locus at 3p21.3 spans approximately 11,000 bp. It contains eight exons, and alternative splicing and usage of two different promoters (Fig. 2A) give rise to eight different transcripts, *RASSF1A-RASSF1H*. Epigenetic inactivation of genes often involves the methylation of CpG islands in their promoters (Hesson et al., 2007). There are two CpG islands associated with the *RASSF1* promoters. A smaller, 737 bp island contains 85 CpGs and spans the promoter for *RASSF1A*, *RASSF1D*, *RASSF1E*, *RASSF1F* and *RASSF1G*. A larger 1365 bp island, containing 139 CpGs, spans the promoter region for *RASSF1B* and *RASSF1C* (Agathanggelou et al., 2005).

RASSF1A is a 340-residue protein that migrates at 39 kDa. It contains a cysteine-rich domain (CRD) reminiscent of the diacylglycerol-binding–CRD domain of Raf-1 towards the N-terminus (residues 50-101), which is not present in the other ubiquitously expressed isoform RASSF1C. Isoforms A-E also contain an RA domain located towards the C-terminus of the protein and a SARAH (Sav-RASSF-Hpo) protein-protein interaction motif at the very C-terminus. A putative ATM phosphorylation site for the DNA repair checkpoint kinase ATM is found in isoforms A, C, D, E and H (Fig. 2B).

Only isoforms A and C have been subjected to extensive biological analysis. Little information is available regarding the functions of splice variants B, D, E, F, G and H. RASSF1C appears to share many of the biological characteristics of RASSF1A and has been implicated as a tumor suppressor in both in vitro and in vivo studies (Li et al., 2004; Vos et al., 2000). However, it has unique functions not shared by RASSF1A, such as coupling DNA damage to the activation of the SAPK-JNK signaling pathway (Kitagawa et al., 2006). RASSF1C and RASSF1A use different promoters, and Latif and co-workers report that RASSF1C is not subject to epigenetic inactivation (Agathanggelou et al., 2005). However, we have observed differential loss of RASSF1C protein in some tumor lines (our unpublished observations). Perhaps the regulation of RASSF1C involves more significant post-transcriptional mechanisms than regulation of RASSF1A.

Table 2. Primary tumors containing *NORE1A* promoter methylation

Tumor type	Frequency*	References
Lung, SCLC	0%	Hesson et al., 2003
Lung, NSCLC	24% 28%	Hesson et al., 2003; Irimia et al., 2004
Hepatocellular Carcinoma	37.5%	Calvisi et al., 2006
Clear cell renal Carcinoma	32%	Chen et al., 2003
Neuroblastoma	3%	Lazcoz et al., 2006
Wilms tumor	15%	Morris et al., 2003

*Frequency of *NORE1A* promoter hypermethylation in tumor type.

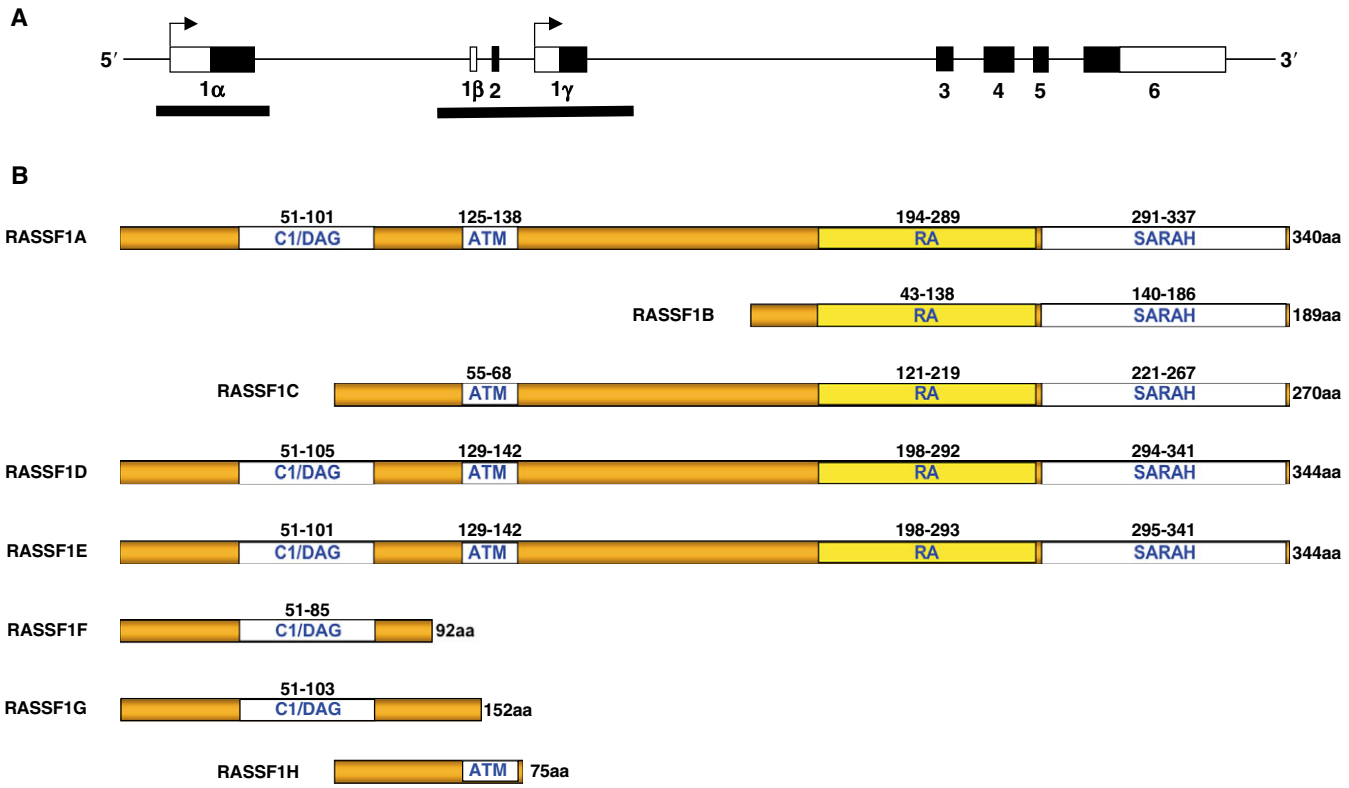

Fig. 2. *RASSF1* gene locus and domain structure of the different RASSF1 isoforms. (A) The *RASSF1* gene locus is characterized by eight exons (boxed regions) and two different promoters (arrows) with two associated CpG islands (black bars). Black boxes represent coding regions and white boxes are non-coding regions. (B) Schematic representation of the different RASSF1 isoforms. C1/DAG, conserved region 1 diacylglycerol-binding domain; ATM, ATM-kinase consensus phosphorylation sequence; RA, RalGDS/AF6 Ras association domain; SARAH, Sav/RASSF/Hpo interaction domain. The position of each domain (as outlined in the Swiss-Prot/TrEMBL database) is indicated above each isoform and the number of amino acids in each isoform is shown on the right.

RASSF1A as a Ras effector

Activated forms of K-Ras, although being transforming oncoproteins, also have growth inhibitory effects, including the induction of apoptosis (Cox and Der, 2003; Downward, 1998). K-Ras must thus have pro-apoptotic effector proteins, which are likely to be downregulated during the development of Ras-dependent tumors. RASSF1A is a pro-apoptotic protein that has a potential RA domain, and so it could mediate some of the pro-apoptoptic effects of K-Ras. This hypothesis is supported by the observation that the related RASSF5 protein can be detected in an endogenous complex with Ras (Vavvas et al., 1998).

The RA domain of RASSF1A can bind to Ras directly in vitro (Vos et al., 2000), and RASSF1A forms a complex with activated K-Ras when overexpressed in cells (Rodriguez-Viciana et al., 2004). Formation of the complex depends on an intact effector domain for Ras and farnesylation of K-Ras (Fig. 3). We have found that K-Ras binds better than H-Ras, even though both share an identical effector domain and both are farnesylated. Other Ras-related proteins also demonstrate the potential to bind RASSF1A, including M-Ras and R-Ras but not Rap (our unpublished observations). M-Ras and R-Ras are post translationally modified by geranylgeranyl, not farnesyl, and this may contribute to the weaker interaction with RASSF1A.

Ortiz-Vega et al., however, have failed to see direct binding between Ras and RASSF1A. They suggest that the interaction is indirect and due to heterodimerization of RASSF1A with RASSF5 (Ortiz-Vega et al., 2002). The use of unfarnesylated Ras in their studies may have led them to underestimate the binding affinity. Confirmation of RASSF1A as a bona fide Ras effector awaits the demonstration that the endogenous proteins form a complex in vivo.

If RASSF1A serves as a pro-apoptotic Ras effector, then one might expect Ras activation to correlate with RASSF1A inactivation in tumors. Several studies have failed to detect such a relationship (Dammann et al., 2003; Li et al., 2003; van Engeland et al., 2002). However, these experiments used the presence or absence of an activating Ras mutation to identify Ras-dependent tumors. In fact, there is a surprisingly poor correlation between the presence of a mutation in the *Ras* gene and the abundance of activated Ras protein in tumor cells (Eckert et al., 2004). Thus, resolving this issue will require direct measurements of Ras-GTP levels in the cells.

Biological functions of RASSF1A

Numerous studies have shown that overexpression of RASSF1A promotes apoptosis, cell cycle arrest and reduces the tumorigenicity of cancer cell lines (for a review, see Agathangelou et al., 2005). RNAi experiments have implicated RASSF1A downregulation in loss of cell cycle control, enhanced genetic instability, enhanced cell motility and

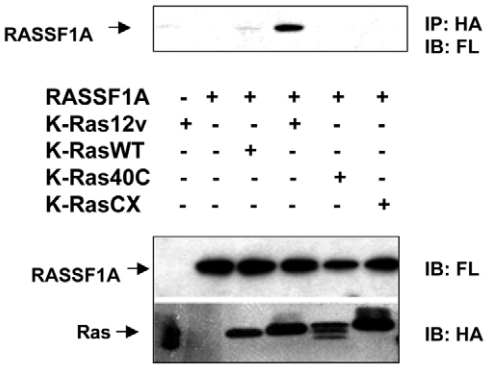

Fig. 3. RASSF1A binds Ras. (A) HEK-293-T cells were transfected with FLAG-tagged RASSF1A and HA-tagged forms of K-Ras12v. The cells were lysed and immunoprecipitated (IP) before being immunoblotted (IB) with HA and FLAG. Upper panel shows immunoprecipitation, lower panel shows protein levels in the cell lysate. Wild-type K-Ras, a Y40C effector mutant of K-Ras12v and a farnesylation-defective mutant of K-Ras12v (K-RasCX) were defective for binding RASSF1A.

resistance to K-Ras and tumor necrosis factor α (TNFα)-induced apoptosis (Baksh et al., 2005; Dallol et al., 2005; Song et al., 2004; Vos et al., 2004; Vos et al., 2006). Thus, RASSF1A appears to regulate multiple biological processes. The mechanisms behind these activities are multifold and remain under investigation but the emerging evidence suggests a role for RASSF1A as a scaffolding protein that can assemble and modulate multiple effector protein complexes.

RASSF1A regulates microtubules

RASSF1A localizes to microtubules and promotes their stabilization (Liu et al., 2003; Dallol et al., 2004; Song et al., 2004; Vos et al., 2004). During interphase, it is localized to cytoplasmic microtubules; during prophase it localizes to centrosomes; during metaphase and anaphase, it localizes to both spindle microtubules and the spindle poles; and it is found at the midzone and midbody during early and late telophase, respectively (Fig. 4).

Microtubules are polymers that continually switch between phases of elongation and shortening; this is known as dynamic instability. Microtubule dynamics can be modulated by a series of microtubule-associated proteins (MAPs) that bind directly to tubulin (Halpain and Dehmelt, 2006). Two-hybrid analysis has identified three such proteins – MAP1b (Dallol et al), C19ORF5 (also known VCY2IP1 or RABP1) (Liu et al., 2002; Song et al., 2005) and MAP4 (G.J.C., unpublished observation) – as direct binding partners of RASSF1A. Thus, RASSF1A could associate with microtubules via MAPs. MAP1b has been shown to promote tubulin polymerization (Togel et al., 1998) and MAP4 has been shown to impede microtubule depolymerization (Nguyen et al., 1998). C19ORF5 has also been shown to enhance microtubule polymerization (Liu et al., 2005; Orbán-Németh et al., 2005). Thus, RASSF1A has the potential to scaffold proteins that we might expect would have a synergistic effect on microtubule polymerization.

We have identified a minimum domain in RASSF1A that is required for the microtubule-stabilizing effects. When this isolated domain is itself overexpressed, it causes a catastrophic collapse of the microtubule network (Vos et al., 2004). The underlying mechanism and whether it involves the direct interaction of RASSF1A with tubulin remains under investigation, but it appears that RASSF1A has the capacity to profoundly influence the dynamic balance of microtubules both positively and negatively.

Maintenance of genomic stability

Genomic instability is one of the hallmarks of transformed cells (Saavedra et al., 2000) and defects in spindle regulation can lead to genomic instability (Wassmann and Benezra, 2001). Since RASSF1A localizes to the centrosome and mitotic spindle, and can modulate tubulin dynamics (Song et al., 2005; Vos et al., 2004; Dallol et al., 2004), it is not surprising that RASSF1A has been implicated in the maintenance of genomic stability (Song et al., 2005; Vos et al., 2004).

C19ORF5 may play a key role in recruiting RASSF1A to the centrosome and spindle. Moreover, inhibition of C19ORF5 expression by RNAi can promote genetic instability similar to

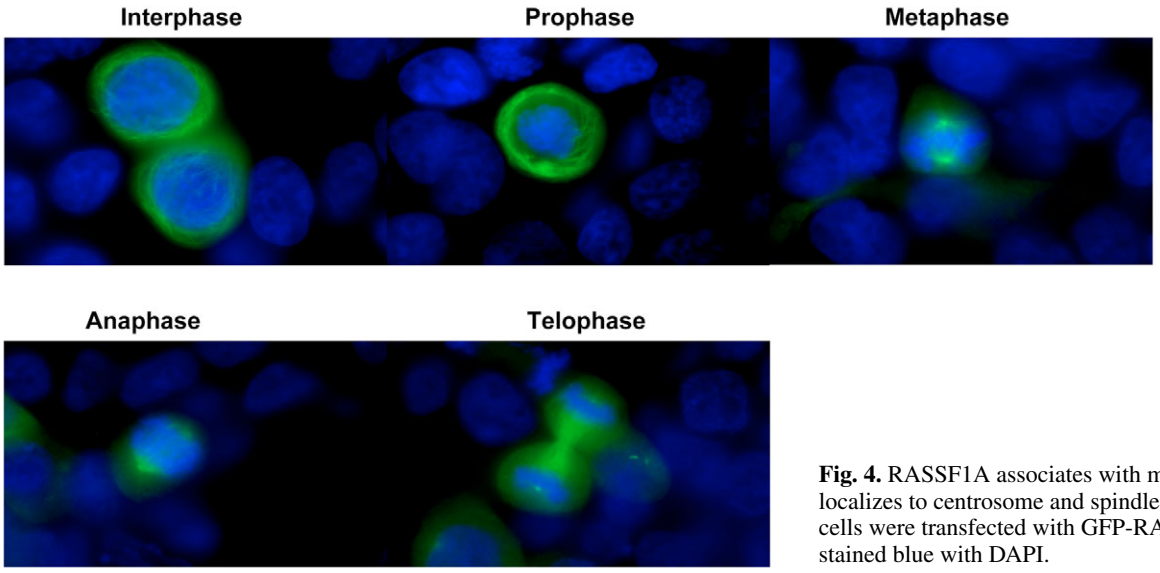

Fig. 4. RASSF1A associates with microtubules and localizes to centrosome and spindles during mitosis. COS cells were transfected with GFP-RASSF1A and the nuclei stained blue with DAPI.

that observed when RASSF1A is downregulated (Song et al., 2005; Dallol et al., 2007). Song et al. suggest that the mechanism of C19ORF5 action is to enhance the ability of RASSF1A to stabilize mitotic cyclins. Thus, loss of function of C19ORF5 leads to premature destruction of mitotic cyclins and accelerated, aberrant mitosis (Song et al., 2004). However, in similar experiments, Dallol et al. observed delayed rather than accelerated mitotic progression and showed a role for C19ORF5 in anchoring α and γ tubulin to the centrosomes (Dallol et al., 2007). This suggests that the abnormalities in sister chromatid separation observed when C19ORF5 is downregulated is due to aberrations in spindle dynamics. This is clearly a complicated issue that may require further experimentation to resolve.

Both RASSF1A and RASSF1C contain a potential ATM kinase (mutated in ataxia telangiectasia) phosphorylation site (Kim et al., 1999). ATM functions as part of the DNA damage checkpoint and has been implicated in regulation of genomic stability (Levitt and Hickson, 2002; Shiloh, 2003). Point mutations that destroy the RASSF1A or RASSF1C ATM phosphorylation site have been found in human tumors (Burbee et al., 2001; Shivakumar et al., 2002). We have been unable to detect any obvious difference in the microtubule-stabilizing activities of wild-type RASSF1A and RASSF1A mutated at the ATM site. However, the equivalent mutant of RASSF1C (S61F) is clearly impaired (Vos et al., 2004). Indeed, this RASSF1C mutant can induce genomic instability at frequencies comparable to those evident in RASSF1A-knockdown studies (our unpublished observation). Thus, RASSF1A and RASSF1C may be mediators through which ATM maintains genomic stability. Moreover, mutant RASSF1C has the potential to serve as an oncogene.

RASSF1A modulates the cell cycle

Initial studies examining the role of RASSF1A in the cell cycle demonstrated a role for RASSF1A at the G1-S checkpoint and showed that RASSF1A modulates the levels of cyclin D1 (Shivakumar et al., 2002). Subsequent work confirmed this and implicated inhibition of the JNK pathway as a mechanism (Whang et al., 2005). RASSF1A could connect to JNK by direct interactions with the kinase MST1 (Khokhlatchev et al., 2002), which can modulate JNK activity (Ura et al., 2007).

RASSF1A could also impact the G1 transition via its direct interaction with the transcription factor $p120^{E4F}$ (Fenton et al., 2004). $p120^{E4F}$ can negatively regulate the transcription of cyclin A2, leading to cell cycle arrest in G1 phase (Fajas et al., 2001). RASSF1A enhances the ability of $p120^{E4F}$ to suppress cyclin A2 and synergizes with $p120^{E4F}$ to induce cell cycle arrest (Fenton et al., 2004; Ahmed-Choudury et al., 2005). However, the role of RASSF1A in cyclin A2 regulation may be complex because it appears to be able to increase cyclin A2 protein levels under some circumstances (Song et al., 2004). Variations in experimental procedures could be responsible for this observed discrepancy. Intriguingly, $p120^{E4F}$ has also been detected at the mitotic spindle and has been implicated in genomic instability (Le Cam et al., 2004). This localization is likely to be mediated by RASSF1A and may indicate a biological role of the RASSF1A-$p120^{E4F}$ interaction that is independent of the latter's transcription factor function.

RASSF1A has been implicated in control of mitotic arrest in prometaphase (Liu et al., 2003; Vos et al., 2004; Rong et al., 2004). An elegant explanation for the M-phase arrest mediated by RASSF1A has been put forward by Song et al., who suggested that it is brought about by the direct interaction of RASSF1A with Cdc20 (Song et al., 2004; Song et al., 2005). Cdc20 is an essential cell cycle regulator required for the completion of mitosis (Yu, 2007). Cdc20 binds and activates the ubiquitin ligase activity of a large molecular machine designated the anaphase-promoting complex (APC). This promotes the ubiquitylation and degradation of cyclins A and B, leading to anaphase and mitotic exit. Song et al. suggest that the interaction with RASSF1A blocks the ability of Cdc20 to activate the APC and that the resultant stabilization of cyclins A and B blocks the mitotic progression that usually follows their degradation (Mathe, 2004; Peters, 2002; Zachariae and Nasmyth, 1999). Liu et al., however, have been unable to confirm the interaction of RASSF1A with Cdc20 (Liu et al., 2007). Thus the role of Cdc20 and APC in RASSF1A-mediated cell cycle control requires further investigation.

Modulation of apoptosis

RASSF family proteins are pro-apoptotic (Vos et al., 2000; Khokhlatchev et al., 2002; Eckfeld et al., 2004; Vos et al., 2003a; Vos et al., 2003b) and several pathways by which RASSF1A may modulate apoptosis have now been identified. MST1 and MST2 are pro-apoptotic serine/threonine kinases that activate the SAPK-JNK signaling pathway and phosphorylate histone H2B (Cheung et al., 2003; Ura et al., 2007). They bind directly to RASSF1A and other RASSF family members via their SARAH motifs (Avruch et al., 2005; Hwang et al., 2007; Khokhlatchev et al., 2002; Oh et al., 2006; Praskova et al., 2004). Consequently, they are obvious pro-apoptotic effectors for RASSF1A. However, the role of RASSF1A in the regulation of MST1 appears complex. In mammalian cells, contradictory effects of RASSF1A on MST1 kinase activity have been reported. Praskova et al. found that MST1 kinase activity is inhibited by RASSF1A whereas Oh et al. and Guo et al. have found that it is activated (Oh et al., 2006; Praskova et al., 2004; Guo et al., 2007). Our own studies support the results of Oh and Guo. Thus, the effects of RASSF1A on MST1 may be context dependent.

RASSF1A also forms a complex with the pro-apoptotic adapter protein CNK1 through interaction with the CRIC and PDZ domains of CNK1 (Rabizadeh et al., 2004). The ability of CNK1 to induce apoptosis appears to require interaction with a RASSF1A-MST1 complex and an as-yet-unidentified effector. Thus, RASSF1A may function as a scaffold for assembly of an apoptotic complex containing CNK1.

Studies in *Drosophila* have recently led to the identification of a pro-apoptotic tumor suppressor kinase cascade. This pathway involves the coupling of MST kinases to the LATs kinases via an adaptor protein called Salvador that acts as a tumor suppressor in *Drosophila* (Harvey and Tapon, 2007). LATs kinases are pro-apoptotic and transgenic mice lacking LATs1 develop tumors (St John et al., 1999; Tao et al., 1999; Yabuta et al., 2000). One target of the LATs kinases that has been identified is the key transcriptional repressor YAP (Huang et al., 2005).

Structural modeling led to the prediction that RASSF1A might bind Salvador through heterodimerization of their SARAH motifs (Scheel and Hofmann, 2003). Although studies in *Drosophila* appeared to show that RASSF1A does not

interact with Salvador (Polesello et al., 2006), recent studies have confirmed that human RASSF1A does bind human Salvador (Guo et al., 2007). Analysis of the protein sequence of the 'RASSF1A' described in *Drosophila* suggests that it is closer to human RASSF5 than to RASSF1A. In our hands, human RASSF5 does not appear to bind Salvador, and this may explain the apparent contradiction. Nevertheless, RASSF1A is therefore connected to the LATs kinase tumor suppressor system via Salvador (Guo et al., 2007; O'Neill et al., 2005). Because RASSF1A can bind Salvador and MST kinases, this is an obvious example of a scaffolding function for RASSF1A.

Bax is a member of the Bcl2 family and an important component of the apoptotic machinery (Sharpe et al., 2004; Tan et al., 2001). Recent work has shown that RASSF1A can also regulate Bax activity (Baksh et al., 2005; Vos et al., 2006). This is accomplished by the direct binding of RASSF1A to modulator of apoptosis-1 (MOAP1), a Bax-binding protein (Tan et al., 2001). The interaction between RASSF1A and MOAP1 is enhanced by activated K-Ras (Vos et al., 2006), and knocking down RASSF1A impairs the ability of oncogenic K-Ras to activate Bax. RASSF1A mutants found in human tumors exhibit impaired interaction with MOAP1, which suggests that subversion of this pathway is important for the development of a tumor. Thus a Ras-RASSF1A-MOAP1 complex appears to be essential for Ras-induced apoptosis. Baksh et al. have observed that RASSF1A expression also enhances TNF-α-induced apoptosis in transformed and non-transformed cells, and this also involves RASSF1A-MOAP1-mediated activation of Bax (Baksh et al., 2005). Activated-Ras-induced and death-receptor-dependent apoptosis might thus both involve RASSF1A-MOAP1-dependent activation of Bax.

Clearly, RASSF1A has the potential to modulate and coordinate multiple pro-apoptotic pathways. It is likely that RASSF1A also interacts with additional apoptotic pathway components.

Cell motility and invasion

The ability of RASSF1A to modulate tubulin dynamics may allow RASSF1A to control cell motility and invasion. Dallol et al. showed that overexpression of RASSF1A inhibits cell migration and alters cell morphology, and knocking down RASSF1A results in reduced cell-cell adhesion (Dallol et al., 2005). We have found that RASSF1A re-expression at physiological levels can impair both the motility and the invasive phenotype of a lung tumor cell line (our unpublished observations). Thus, loss of RASSF1A function may contribute to the later stages of tumorigenesis as well as the early stages.

Other potential RASSF1A functions and interactions

In addition to these fairly well characterized functions, RASSF1A may have other functions within the cell. It interacts with plasma membrane calmodulin-dependent Ca^{2+} ATPase (PMCA), an enzyme involved in the transport of Ca^{2+} out of the cell (Armesilla et al., 2004), which may indicate a connection with Ca^{2+} signaling.

Microarray studies have shown that RASSF1A can impact the activity of a number of genes involved in transcription, signal transduction, protein synthesis, cell cycle regulation, metabolism, and cell adhesion (Agathanggelou et al., 2003; Chow et al., 2006). The different studies identified distinct groups of target genes, which is probably a reflection of the different cell types used. Further studies will clearly be required to reveal the exact nature of the involvement of RASSF1A in these pathways.

Conclusion

The RASSF1A protein modulates a broad range of cellular functions that are essential for normal growth control. *RASSF1A* expression is lost in a wide variety of human tumors by silencing resulting primarily from promoter hypermethylation. The high frequency with which *RASSF1A* is silenced in tumors suggests that it plays a pivotal role in the development of human cancer.

Lacking enzymatic activity, the RASSF1A protein appears to serve as a node that can scaffold multiple tumor suppressor pathways. Those pathways known to contain potential effectors of RASSF1A function are shown in Fig. 1. There are almost certainly others, and the role of the Salvador-LATs pathway in RASSF1A is of particular current interest. All of these pathways have the potential to be modified by Ras, although the physiological interaction between Ras and RASSF1A has yet to be confirmed. RASSF1A represents an important potential diagnostic and therapeutic target. Because the gene remains intact but dormant in most tumors, reactivation by promoter demethylation would present a novel approach to therapy.

References

Agathanggelou, A., Honorio, S., Macartney, D. P., Martinez, A., Dallol, A., Rader, J., Fullwood, P., Chauhan, A., Walker, R., Shaw, J. A. et al. (2001). Methylation associated inactivation of RASSF1A from region 3p21.3 in lung, breast and ovarian tumors. *Oncogene* **20**, 1509-1518.

Agathanggelou, A., Bièche, I., Ahmed-Choudhury, J., Nicke, B., Dammann, R., Baksh, S., Gao, B., Minna, J. D., Downward, J., Maher, E. R. et al. (2003). Identification of novel gene expression targets for the Ras association domain family 1 (*RASSF1A*) tumor suppressor gene in non-small cell lung cancer and neuroblastoma. *Cancer Res.* **63**, 5344-5351.

Agathanggelou, A., Cooper, W. N. and Latif, F. (2005). Role of the Ras-association domain family 1 tumor suppressor gene in human cancers. *Cancer Res.* **65**, 3497-3508.

Ahmed-Choudhury, J., Agathanggelou, A., Fenton, S. L., Ricketts, C., Clark, G. J., Maher, E. R. and Latif, F. (2005). Transcriptional regulation of cyclin A2 by RASSF1A through the enhanced binding of $p120^{E4F}$ to the cyclin A2 promoter. *Cancer Res.* **65**, 2690-2697.

Akino, K., Toyota, M., Suzuki, H., Mita, H., Sasaki, Y., Ohe-Toyota, M., Issa, J. P., Hinoda, Y., Imai, K. and Tokino, T. (2005). The Ras effector RASSF2 is a novel tumor-suppressor gene in human colorectal cancer. *Gastroenterology* **129**, 156-169.

Allen, N. P., Donninger, H., Vos, M. D., Eckfeld, K., Hesson, L., Gorden, L., Birrer, M. J., Latif, F. and Clark, G. J. (2007). RASSF6 is a novel member of the RASSF family of tumor suppressors. *Oncogene* doi:10.1038/sj.onc.1210440.

Armesilla, A. L., Williams, J. C., Buch, M. H., Pickard, A., Emerson, M., Cartwright, E. J., Oceandy, D., Vos, M. D., Gillies, S., Clark, G. J. et al. (2004). Novel functional interaction between the plasma membrane Ca^{2+} pump 4b and the proapoptotic tumor suppressor Ras-associated factor 1 (RASSF1). *J. Biol. Chem.* **279**, 31318-31328.

Astuti, D., Agathanggelou, A., Honorio, S., Dallol, A., Martinsson, T., Kogner, P., Cummins, C., Neumann, H. P., Voutilainen, R., Dahia, P. et al. (2001). RASSF1A promoter region CpG island hypermethylation in phaeochromocytomas and neuroblastoma tumours. *Oncogene* **20**, 7573-7577.

Avruch, J., Praskova, M., Ortiz-Vega, S., Liu, M. and Zhang, X. F. (2005). Nore1 and RASSF1 regulation of cell proliferation and of the MST1/2 kinases. *Meth. Enzymol.* **407**, 290-310.

Baksh, S., Tommasi, S., Fenton, S., Yu, V. C., Martins, M., Pfeifer, G. P., Latif, F., Downward, J. and Neel, B. G. (2005). The tumor suppressor RASSF1A and MAP-1 link death receptor signaling to Bax conformational change and cell death. *Mol. Cell* **18**, 637-650.

Burbee, D. G., Forgacs, E., Zöchbauer-Müller, S., Shivakumar, L., Fong, K., Gao, B., Randle, D., Kondo, M., Virmani, A., Bader, S. et al. (2001). Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. *J. Natl. Cancer Inst.* **93**, 691-699.

Calvisi, D. F, Ladu, S., Gorden, A., Farina, M., Conner, E. A., Lee, J.-S., Factor, V. M. and Thorgeirsson, S. S. (2006). Ubiquitous activation of *Ras* and Jak/Stat pathways in human HCC. *Gastroenterology* **130**, 1117-1128.

Chan, M. W., Chan, L. W., Tang, N. L., Lo, K. W., Tong, J. H., Chan, A. W., Cheung, H. Y., Wong, W. S., Chan, P. S., Lai, F. M. et al. (2003). Frequent hypermethylation of promoter region of RASSF1A in tumor tissues and voided urine of urinary bladder cancer patients. *Int. J. Cancer* **104**, 611-616.

Chen, H., Suzuki, M., Nakamura, Y., Ohira, M., Ando, S., Iida, T., Nakajima, T., Nakagawara, A. and Kimura, H. (2006). Aberrant methylation of RASGRF2 and RASSF1A in human non-small cell lung cancer. *Oncol. Rep.* **15**, 1281-1285.

Chen, J., Lui, W. O., Vos, M. D., Clark, G. J., Takahashi, M., Schoumans, J., Khoo, S. K., Petillo, D., Lavery, T., Sugimura, J. et al. (2003). The t(1;3) breakpoint-spanning genes LSAMP and NORE1 are involved in clear cell renal cell carcinomas. *Cancer Cell* **4**, 405-413.

Cheung, W. L., Ajiro, K., Samejima, K., Kloc, M., Cheung, P., Mizzen, C. A., Beeser, A., Etkin, L. D., Chernoff, J., Earnshaw, W. C. et al. (2003). Apoptotic phosphorylation of histone H2B is mediated by mammalian sterile twenty kinase. *Cell* **113**, 507-517.

Chow, L. S., Lam, C. W., Chan, S. Y., Tsao, S. W., To, K. F., Tong, S. F., Hung, W. K., Dammann, R., Huang, D. P. and Lo, K. W. (2006). Identification of RASSF1A modulated genes in nasopharyngeal carcinoma. *Oncogene* **25**, 310-316.

Cohen, Y., Singer, G., Lavie, O., Dong, S. M., Beller, U. and Sidransky, D. (2003). The RASSF1A tumor suppressor gene is commonly inactivated in adenocarcinoma of the uterine cervix. *Clin. Cancer Res.* **9**, 2981-2984.

Cox, A. D. and Der, C. J. (2003). The dark side of Ras: regulation of apoptosis. *Oncogene* **22**, 8999-9006.

Dallol, A., Agathanggelou, A., Fenton, S. L., Ahmed-Choudhury, J., Hesson, L., Vos, M. D., Clark, G. J., Downward, J., Maher, E. R. and Latif, F. (2004). RASSF1A interacts with microtubule-associated proteins and modulates microtubule dynamics. *Cancer Res.* **64**, 4112-4116.

Dallol, A., Agathanggelou, A., Tommasi, S., Pfeifer, G. P., Maher, E. R. and Latif, F. (2005). Involvement of the *RASSF1A* tumor suppressor gene in controlling cell migration. *Cancer Res.* **65**, 7653-7659.

Dallol, A, Cooper, W. N., Al-Mulla, F., Agathanggelou, A., Maher, E. R. and Latif, F. (2007). Depletion of the Ras association domain family 1, isoform A-associated novel microtubule-associated protein, C19ORF5/MAP1S, causes mitotic abnormalities. *Cancer Res*. **67**, 492-500.

Dammann, R., Li, C., Yoon, J.-H., Chin, P. L., Bates, S. and Pfeifer, G. P. (2000). Epigenetic inactivation of a RAS association domain family protein from lung tumour suppressor locus 3p21.3. *Nat. Genet.* **25**, 315-319.

Dammann, R., Schagdarsurengin, U., Strunnikova, M., Rastetter, M., Seidel, C., Liu, L., Tommasi, S. and Pfeifer, G. P. (2003). Epigenetic inactivation of the Ras-association domain family 1 (RASSF1A) gene and its function in human carcinogenesis. *Histol. Histopathol.* **18**, 665-677.

Dong, S. M., Sun, D. I., Benoit, N. E., Kuzmin, I., Lerman, M. I. and Sidransky, D. (2003). Epigenetic inactivation of RASSF1A in head and neck cancer. *Clin. Cancer Res.* **9**, 3635-3640.

Downward, J. (1998). Ras signalling and apoptosis. *Curr. Opin. Genet. Dev.* **8**, 49-54.

Dreijerink, K., Braga, E., Kuzmin, I., Geil, L., Duh, F.-M., Angeloni, D., Zbar, B., Lerman, M. I., Stanbridge, E. J., Minna, J. D. et al. (2001). The candidate tumor suppressor gene, *RASSF1A*, from human chromosome 3p21.3 is involved in kidney tumorigenesis. *Proc. Natl. Acad. Sci. USA* **98**, 7504-7509.

Eckert, L. B., Repasky, G. A., Ulku, A. S., McFall, A., Zhou, H., Sartor, C. I. and Der, C. J. (2004). Involvement of Ras activation in human breast cancer cell signaling, invasion, and anoikis. *Cancer Res.* **64**, 4585-4592.

Eckfeld, K., Hesson, L., Vos, M. D., Bièche, I., Latif, F. and Clark, G. J. (2004). RASSF4/AD037 is a potential ras effector/tumor suppressor of the RASSF family. *Cancer Res.* **64**, 8688-8693.

Endoh, M., Tamura, G., Honda, T., Homma, N., Terashima, M., Nishizuka, S. and Motoyama, T. (2005). RASSF2, a potential tumour suppressor, is silenced by CpG island hypermethylation in gastric cancer. *Br. J. Cancer* **93**, 1395-1399.

Fajas, L., Paul, C., Vie, A., Estrach, S., Medema, R., Blanchard, J. M., Sardet, C. and Vignais, M. L. (2001). Cyclin A is a mediator of $p120^{E4F}$-dependent cell cycle arrest in G1. *Mol. Cell. Biol.* **21**, 2956-2966.

Falvella, F. S., Manenti, G., Spinola, M., Pignatiello, C., Conti, B., Pastorino, U. and Dragani, T. A. (2006). Identification of RASSF8 as a candidate lung tumor suppressor gene. *Oncogene* **25**, 3934-3938.

Fenton, S. L., Dallol, A., Agathanggelou, A., Hesson, L., Ahmed-Choudhury, J., Baksh, S., Sardet, C., Dammann, R., Minna, J. D., Downward, J. et al. (2004). Identification of the E1A-regulated transcription factor $p120^{E4F}$ as an interacting partner of the RASSF1A candidate tumor suppressor gene. *Cancer Res.* **64**, 102-107.

Grote, H. J., Schmiemann, V., Geddert, H., Bocking, A., Kappes, R., Gabbert, H. E. and Sarbia, M. (2006). Methylation of RAS association domain family protein 1A as a biomarker of lung cancer. *Cancer* **108**, 129-134.

Guo, C., Tommasi, S., Liu, L., Yee, J. K., Dammann, R. and Pfeifer, G. P. (2007). RASSF1A is part of a complex similar to the Drosophila Hippo/Salvador/Lats tumor-suppressor network. *Curr. Biol.* **17**, 700-705.

Halpain, S. and Dehmelt, L. (2006). The MAP1 family of microtubule-associated proteins. *Genome Biol*. **7**, 224.

Hamilton, D. W., Lusher, M. E., Lindsey, J. C., Ellison, D. W. and Clifford, S. C. (2005). Epigenetic inactivation of the RASSF1A tumour suppressor gene in ependymoma. *Cancer Lett.* **227**, 75-81.

Harada, K., Toyooka, S., Maitra, A., Maruyama, R., Toyooka, K. O., Timmons, C. F., Tomlinson, G. E., Mastrangelo, D., Hay, R. J., Minna, J. D. et al. (2002). Aberrant promoter methylation and silencing of the RASSF1A gene in pediatric tumors and cell lines. *Oncogene* **21**, 4345-4349.

Harvey, K. and Tapon, N. (2007). The Salvador-Warts-Hippo pathway – an emerging tumour-suppressor network. *Nat. Rev. Cancer* **7**, 182-191.

Hesson, L., Dallol, A., Minna, J. D., Maher, E. R. and Latif, F. (2003). NORE1A, a homologue of RASSF1A tumour suppressor gene is inactivated in human cancers. *Oncogene* **22**, 947-954.

Hesson, L., Bièche, I., Krex, D., Criniere, E., Hoang-Xuan, K., Maher, E. R. and Latif, F. (2004). Frequent epigenetic inactivation of RASSF1A and BLU genes located within the critical 3p31.3 region in gliomas. *Oncogene* **23**, 2408-2419.

Hesson, L. B., Wilson, R., Morton, D., Adams, C., Walker, M., Maher, E. R. and Latif, F. (2005). CpG island promoter hypermethylation of a novel Ras-effector gene RASSF2A is an early event in colon carcinogenesis and correlates inversely with K-ras mutations. *Oncogene* **24**, 3987-3994.

Hesson, L. B., Cooper, W. N. and Latif, F. (2007). The role of RASSF1A methylation in cancer. *Dis. Markers* **23**, 73-87.

Hogg, R. P., Honorio, S., Martinez, A., Agathanggelou, A., Dallol, A., Fullwood, P., Weichselbaum, R., Kuo, M. J., Maher, E. R. and Latif, F. (2002). Frequent 3p allele loss and epigenetic inactivation of the RASSF1A tumour suppressor gene from region 3p21.3 in head and neck squamous cell carcinoma. *Eur. J. Cancer* **38**, 1585-1592.

Honorio, S., Agathanggelou, A., Wernert, N., Rothe, M., Maher, E. R. and Latif, F. (2003). Frequent epigenetic inactivation of the RASSF1A tumour suppressor gene in testicular tumours and distinct methylation profiles of seminoma and nonseminoma testicular germ cell tumours. *Oncogene* **22**, 461-466.

Horiguchi, K., Tomizawa, Y., Tosaka, M., Ishiuchi, S., Kurihara, H., Mori, M. and Saito, N. (2003). Epigenetic inactivation of RASSF1A candidate tumor suppressor gene at 3p21.3 in brain tumors. *Oncogene* **22**, 7862-7865.

Huang, J., Wu, S., Barrera, J., Matthews, K. and Pan, D. (2005). The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. *Cell* **122**, 421-434.

Hull, J., Rowlands, K., Lockhart, E., Sharland, M., Moore, C., Hanchard, N. and Kwiatkowski, D. P. (2004). Haplotype mapping of the bronchiolitis susceptibility locus near IL8. *Hum. Genet.* **114**, 272-279.

Hwang, E., Ryu, K. S., Paakkonen, K., Guntert, P., Cheong, H. K., Lim, D. S., Lee, J. O., Jeon, Y. H. and Cheong, C. (2007). Structural insight into dimeric interaction of the SARAH domains from Mst1 and RASSF family proteins in the apoptosis pathway. *Proc. Natl. Acad. Sci. USA* **104**, 9236-9241.

Irimia, M., Fraga, M. F., Sanchez-Cespedes, M. and Esteller, M. (2004). CpG island promoter hypermethylation of the Ras-effector gene NORE1A occurs in the context of a wild-type K-ras in lung cancer. *Oncogene* **23**, 8695-8699.

Jeronimo, C., Henrique, R., Hoque, M. O., Mambo, E., Ribeiro, F. R., Varzim, G., Oliveira, J., Teixeira, M. R., Lopes, C. and Sidransky, D. (2004). A quantitative promoter methylation profile of prostate cancer. *Clin. Cancer Res.* **10**, 8472-8478.

Johan, M. F., Bowen, D. T., Frew, M. E., Goodeve, A. C. and Reilly, J. T. (2005). Aberrant methylation of the negative regulators RASSFIA, SHP-1 and SOCS-1 in myelodysplastic syndromes and acute myeloid leukaemia. *Br. J. Haematol.* **129**, 60-65.

Katagiri, K., Maeda, A., Shimonaka, M. and Kinashi, T. (2003). RAPL, a Rap1-binding molecule that mediates Rap1-induced adhesion through spatial regulation of LFA-1. *Nat. Immunol.* **4**, 741-748.

Katoh, H., Shibata, T., Kokubu, A., Ojima, H., Fukayama, M., Kanai, Y. and Hirohashi, S. (2006). Epigenetic instability and chromosomal instability in hepatocellular carcinoma. *Am. J. Pathol.* **168**, 1375-1384.

Khokhlatchev, A., Rabizadeh, S., Xavier, R., Nedwidek, M., Chen, T., Zhang, X. F., Seed, B. and Avruch, J. (2002). Identification of a novel Ras-regulated proapoptotic pathway. *Curr. Biol.* **12**, 253-265.

Kim, S. T., Lim, D.-S., Canman, C. E. and Kastan, M. B. (1999). Substrate specificities and identification of putative substrates of ATM kinase family members. *J. Biol. Chem.* **274**, 37538-37543.

Kitagawa, D., Kajiho, H., Negishi, T., Ura, S., Watanabe, T., Wada, T., Ichijo, H., Katada, T. and Nishina, H. (2006). Release of RASSF1C from the nucleus by Daxx degradation links DNA damage and SAPK/JNK activation. *EMBO J.* **25**, 3286-3297.

Knudson, A. G., Jr (1971). Mutation and cancer: statistical study of retinoblastoma. *Proc. Natl. Acad. Sci. USA* **68**, 820-823.

Kuzmin, I., Gillespie, J. W., Protopopov, A., Geil, L., Dreijerink, K., Yang, Y., Vocke, C. D., Duh, F.-M., Zabarovsky, E., Minna, J. D. et al. (2002). The RASSF1A tumor suppressor gene is inactivated in protate tumors and suppresses growth of prostate carcinoma cell lines. *Cancer Res.* **62**, 3498-3502.

Lambros, M. B., Fiegler, H., Jones, A., Gorman, P., Roylance, R. R., Carter, N. P. and Tomlinson, I. P. (2005). Analysis of ovarian cancer cell lines using array-based comparative genomic hybridization. *J. Pathol.* **205**, 29-40.

Lazcoz, P., Munoz, J., Nistal, M., Pestana, A., Encio, I. and Castresana, J. S. (2006). Frequent promoter hypermethylation of RASSF1A and CASP8 in neuroblastoma. *BMC Cancer* **6**, 254.

Le Cam, L., Lacroix, M., Ciemerych, M. A., Sardet, C. and Sicinski, P. (2004). The E4F protein is required for mitotic progression during embryonic cell cycles. *Mol. Cell. Biol*. **24**, 6467-6475.

Lerman, M. I. and Minna, J. D. (2000). The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. *Cancer Res.* **60**, 6116-6133.

Levitt, N. C. and Hickson, I. D. (2002). Caretaker tumour suppressor genes that defend genome integrity. *Trends Mol. Med.* **8**, 179-186.

Li, J., Zhang, Z., Dai, Z., Popkie, A. P., Plass, C., Morrison, C., Wang, Y. and You, M. (2003). RASSF1A promoter methylation and Kras2 mutations in non small cell lung cancer. *Neoplasia* **5**, 362-366.

Li, J., Wang, F., Protopopov, A., Malyukova, A., Kashuba, V., Minna, J. D., Lerman, M. I., Klein, G. and Zabarovsky, E. (2004). Inactivation of RASSF1C during in vivo tumor growth identifies it as a tumor suppressor gene. *Oncogene* **23**, 5941-5949.

Liu, L., Amy, V., Liu, G. and McKeehan, W. L. (2002). Specificity of the methylation-suppressed A isoform of candidate tumor suppressor RASSF1 for microtubule

hyperstabilization is determined by cell death inducer C19ORF5. *Cancer Res.* **65**, 1830-1838.

Liu, L., Tommasi, S., Lee, D. H., Dammann, R. and Pfeifer, G. P. (2003). Control of microtubule stability by the RASSF1A tumor suppressor. *Oncogene* **22**, 8125-8136.

Liu, L., Broaddus, R. R., Yao, J. C., Xie, S., White, J. A., Wu, T. T., Hamilton, S. R. and Rashid, A. (2005). Epigenetic alterations in neuroendocrine tumors: methylation of RAS-association domain family 1, isoform A and p16 genes are associated with metastasis. *Mod. Pathol.* **18**, 1632-1640.

Liu, L., Baier, K., Dammann, R. and Pfeifer, G. P. (2007). The tumor suppressor RASSF1A does not interact with Cdc20, an activator of the anaphase-promoting complex. *Cell Cycle* **6**, 1663-1665.

Lusher, M. E., Lindsey, J. C., Latif, F., Pearson, A. D. J., Ellison, D. W. and Clifford, S. C. (2002). Biallelic epigenetic inactivation of the *RASSF1A* tumor suppressor gene in medulloblastoma development. *Cancer Res.* **62**, 5906-5911.

Macheiner, D., Heller, G., Kappel, S., Bichler, C., Stattner, S., Ziegler, B., Kandioler, D., Wrba, F., Schulte-Hermann, R., Zöchbauer-Müller, S. et al. (2006). NORE1B, a candidate tumor suppressor, is epigenetically silenced in human hepatocellular carcinoma. *J. Hepatol.* **45**, 81-89.

Makarla, P. B., Saboorian, M. H., Ashfaq, R., Toyooka, K. O., Toyooka, S., Minna, J. D., Gazdar, A. F. and Schorge, J. O. (2005). Promoter hypermethylation profile of ovarian epithelial neoplasms. *Clin. Cancer Res.* **11**, 5365-5369.

Marsit, C. J., Karagas, M. R., Schned, A. and Kelsey, K. T. (2006). Carcinogen exposure and epigenetic silencing in bladder cancer. *Ann. N. Y. Acad. Sci.* **1076**, 810-821.

Mathe, E. (2004). RASSF1A, the new guardian of mitosis. *Nat. Genet.* **36**, 117-118.

Michalowski, M. B., de Fraipont, F., Michelland, S., Entz-Werle, N., Grill, J., Pasquier, B., Favrot, M. C. and Plantaz, D. (2006). Methylation of RASSF1A and TRAIL pathway-related genes is frequent in childhood intracranial ependymomas and benign choroid plexus papilloma. *Cancer Genet. Cytogenet.* **166**, 74-81.

Miranda, E., Destro, A., Malesci, A., Balladore, E., Bianchi, P., Baryshnikova, E., Franchi, G., Morenghi, E., Laghi, L., Gennari, L. et al. (2006). Genetic and epigenetic changes in primary metastatic and nonmetastatic colorectal cancer. *Br. J. Cancer* **95**, 1101-1107.

Morris, M. R., Hesson, L. B., Wagner, K. J., Morgan, N. V., Astuti, D., Lees, R. D., Cooper, W. N., Lee, J., Gentle, D., Macdonald, F. et al. (2003). Multigene methylation analysis of Wilms' tumour and adult renal cell carcinoma. *Oncogene* **22**, 6794-6801.

Murray, P. G., Qiu, G. H., Fu, L., Waites, E. R., Srivastava, G., Heys, D., Agathanggelou, A., Latif, F., Grundy, R. G., Mann, J. R. et al. (2004). Frequent epigenetic inactivation of the RASSF1A tumor suppressor gene in Hodgkin's lymphoma. *Oncogene* **23**, 1326-1331.

Nakamura, N., Carney, J. A., Jin, L., Kajita, S., Pallares, J., Zhang, H., Qian, X., Sebo, T. J., Erickson, L. A. and Lloyd, R. V. (2005). RASSF1A and NORE1A methylation and BRAFV600E mutations in thyroid tumors. *Lab. Invest.* **85**, 1065-1075.

Nguyen, H. L, Gruber, D., Mcgraw, T., Sheetz, M. P. and Bulinski, J. C. (1998). Stabilization and functional modulation of microtubules by microtubule-associated protein 4. *Biol. Bull.* **194**, 354-357.

Oh, H. J., Lee, K. K., Song, S. J., Jin, M. S., Song, M. S., Lee, J. H., Im, C. R., Lee, J. O., Yonehara, S. and Lim, D.-S. (2006). Role of the tumor suppressor RASSF1A in Mst1-mediated apoptosis. *Cancer Res.* **66**, 2562-2569.

Oliveira, C., Velho, S., Domingo, E., Preto, A., Hofstra, R. M., Hamelin, R., Yamamoto, H., Seruca, R. and Schwartz, S., Jr (2005). Concomitant RASSF1A hypermethylation and KRAS/BRAF mutations occur preferentially in MSI sporadic colorectal cancer. *Oncogene* **24**, 7630-7634.

O'Neill, E. E., Matallanas, D. and Kolch, W. (2005). Mammalian sterile 20-like kinases in tumor suppression: an emerging pathway. *Cancer Res.* **65**, 5485-5487.

Orbán-Németh, Z., Simader, H., Badurek, S., Tranciková, A. and Propst, F. (2006). Differences in the regulation of microtubule stability by the pro-rich region variants of microtubule-associated protein 4. *FEBS Lett.* **580**, 3505-3510.

Ortiz-Vega, S., Khokhlatchev, A., Nedwidek, M., Zhang, X. F., Dammann, R., Pfeifer, G. P. and Avruch, J. (2002). The putative tumor suppressor RASSF1A homodimerizes and heterodimerizes with the Ras-GTP binding protein Nore1. *Oncogene* **21**, 1381-1390.

Pan, Z. G., Kashuba, V. I., Liu, X. Q., Shao, J. Y., Zhang, R. H., Jiang, J. H., Guo, C., Zabarovsky, E., Ernberg, I. and Zeng, Y. X. (2005). High frequency somatic mutations in RASSF1A in nasopharyngeal carcinoma. *Cancer Biol. Ther.* **4**, 1116-1122.

Peters, J. M. (2002). The anaphase-promoting complex: proteolysis in mitosis and beyond. *Mol. Cell* **9**, 931-943.

Pfeifer, G. P. and Dammann, R. (2005). Methylation of the tumor suppressor gene *RASSF1A* in human tumors. *Biochemistry Mosc.* **70**, 576-583.

Polesello, C., Huelsmann, S., Brown, N. H. and Tapon, N. (2006). The Drosophila RASSF homolog antagonizes the hippo pathway. *Curr. Biol.* **16**, 2459-2465.

Praskova, M., Khokhlatchev, A., Ortiz-Vega, S. and Avruch, J. (2004). Regulation of the MST1 kinase by autophosphorylation, by the growth inhibitory proteins RASSF1 and NORE1, and by Ras. *Biochem. J.* **381**, 453-462.

Rabizadeh, S., Xavier, R. J., Ishiguro, K., Bernabeortiz, J., Lopez-Ilasaca, M., Khokhlatchev, A., Mollahan, P., Pfeifer, G. P., Avruch, J. and Seed, B. (2004). The scaffold protein CNK1 interacts with the tumor suppressor RASSF1A and augments RASSF1A-induced cell death. *J. Biol. Chem.* **279**, 29247-29254.

Rodriguez-Viciana, P., Sabatier, C. and McCormick, F. (2004). Signaling specificity by Ras family GTPases is determined by the full spectrum of effectors they regulate. *Mol. Cell. Biol.* **24**, 4943-4954.

Rong, R., Jin, W., Zhang, J., Sheikh, M. S. and Huang, Y. (2004). Tumor suppressor RASSF1A is a microtubule-binding protein that stabilizes microtubules and induces G2/M arrest. *Oncogene* **23**, 8216-8230.

Saavedra, H. I., Knauf, J. A., Shirokawa, J. M., Wang, J., Ouyang, B., Elisei, R., Stambrook, P. J. and Fagin, J. A. (2000). The RAS oncogene induces genomic instability in thyroid PCCL3 cell via the MAPK pathway. *Oncogene* **19**, 3948-3954.

Safar, A. M., Spencer, H., III, Su, X., Coffey, M., Cooney, C. A., Ratnasinghe, L. D., Hutchins, L. F. and Fan, C. Y. (2005). Methylation profiling of archived non-small cell lung cancer: a promising prognostic system. *Clin. Cancer Res.* **11**, 4400-4405.

Schagdarsurengin, U., Gimm, O., Dralle, H., Hoang-Vu, C. and Dammann, R. (2006). CpG island methylation of tumor-related promoters occurs preferentially in undifferentiated carcinoma. *Thyroid* **16**, 633-642.

Scheel, H. and Hofmann, K. (2003). A novel interaction motif, SARAH, connects three classes of tumor suppressor. *Curr. Biol.* **13**, R899-R900.

Sharpe, J. C., Arnoult, D. and Youle, R. J. (2004). Control of mitochondrial permeability by Bcl-2 family members. *Biochim. Biophys. Acta* **1644**, 107-113.

Shiloh, Y. (2003). ATM and related protein kinases: safeguarding genome integrity. *Nat. Rev. Cancer* **3**, 155-168.

Shinozaki, M., Hoon, D. S., Giuliano, A. E., Hansen, N. M., Wang, H. J., Turner, R. and Taback, B. (2005). Distinct hypermethylation profile of primary breast cancer is associated with sentinel lymph node metastasis. *Clin. Cancer Res.* **11**, 2156-2162.

Shivakumar, L., Minna, J., Sakamaki, T., Pestell, R. and White, M. A. (2002). The RASSF1A tumor suppressor blocks cell cycle progression and inhibits cyclin D1 accumulation. *Mol. Cell. Biol.* **22**, 4309-4318.

Song, M. S., Song, S. J., Ayad, N. G., Chang, J. S., Lee, J. H., Hong, H. K., Lee, H., Choi, N., Kim, J., Kim, H. et al. (2004). The tumor suppressor RASSF1A regulates mitosis by inhibiting the APC-Cdc20 complex. *Nat. Cell Biol.* **6**, 129-137.

Song, M. S., Chang, J. S., Song, S. J., Yang, T. H., Lee, H. and Lim, D.-S. (2005). The centrosomal protein RAS association domain family protein 1A (RASSF1A)-binding protein 1 regulates mitotic progression by recruiting RASSF1A to spindle poles. *J. Biol. Chem.* **280**, 3920-3927.

Spugnardi, M., Tommasi, S., Dammann, R., Pfeifer, G. P. and Hoon, D. S. (2003). Epigenetic inactivation of RAS association domain family protein 1 (RASSF1A) in malignant cutaneous melanoma. *Cancer Res.* **63**, 1639-1643.

St John, M. A., Tao, W., Fei, X., Fukumoto, R., Carcangiu, M. L., Brownstein, D. G., Parlow, A. F., McGrath, J. and Xu, T. (1999). Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction. *Nat. Genet.* **21**, 182-186.

Tan, K. O., Tan, K. M., Chan, S. L., Yee, K. S., Bevort, M., Ang, K. C. and Yu, V. C. (2001). MAP-1, a novel proapoptotic protein containing a BH3-like motif that associates with Bax through its Bcl-2 homology domains. *J. Biol. Chem.* **276**, 2802-2807.

Tan, S. H., Ida, H., Goh, B. C., Hsieh, W., Loh, M. and Ito, Y. (2006). Analyses of promoter hypermethylation for RUNX3 and other tumor suppressor genes in nasopharyngeal carcinoma. *Anticancer Res.* **26**, 4287-4292.

Tao, W., Zhang, S., Turenchalk, G. S., Stewart, R. A., St John, M. A., Chen, W. and Xu, T. (1999). Human homologue of the Drosophila melanogaster lats tumour suppressor modulates CDC2 activity. *Nat. Genet.* **21**, 177-181.

Teodoridis, J. M., Hall, J., Marsh, S., Kannall, H. D., Smyth, C., Curto, J., Siddiqui, N., Gabra, H., McLeod, H. L., Strathdee, G. et al. (2005). CpG island methylation of DNA damage response genes in advanced ovarian cancer. *Cancer Res.* **65**, 8961-8967.

Tischoff, I., Markwarth, A., Witzigmann, H., Uhlmann, D., Hauss, J., Mirmohammadsadegh, A., Wittekind, C., Hengge, U. R. and Tannapfel, A. (2005). Allele loss and epigenetic inactivation of 3p21.3 in malignant liver tumors. *Int. J. Cancer* **115**, 684-689.

Togel, M., Wiche, G. and Propst, F. (1998). Novel features of the light chain of microtubule-associated protein MAP1B: microtubule stabilization, self interaction, actin filament binding, and regulation by the heavy chain. *J. Cell Biol.* **143**, 695-707.

Tomizawa, Y., Kohno, T., Kondo, H., Otsuka, A., Nishioka, M., Niki, T., Yamada, T., Maeshima, A., Yoshimura, K., Saito, R. et al. (2002). Clinicopathological significance of epigenetic inactivation of *RASSF1A* at 3p21.3 in stage I lung adenocarcinoma. *Clin. Cancer Res.* **8**, 2362-2368.

Tommasi, S., Dammann, R., Jin, S. G., Zhang, X. F., Avruch, J. and Pfeifer, G. P. (2002). RASSF3 and NORE1: identification and cloning of two homologues of the putative tumor suppressor gene RASSF1. *Oncogene* **21**, 2713-2720.

Tommasi, S., Dammann, R., Zhang, Z., Wang, Y., Liu, L., Tsark, W. M., Wilczynski, S. P., Li, J., You, M. and Pfeifer, G. P. (2005). Tumor susceptibility of *RASSF1A* knockout mice. *Cancer Res.* **65**, 92-98.

Ura, S., Nishina, H., Gotoh, Y. and Katada, T. (2007). Activation of the JNK pathway by MST1 is essential and sufficient for the induction of chromatin condensation during apoptosis. *Mol. Cell. Biol.* **27**, 5514-5522.

van der Weyden, L., Tachibana, K. K., Gonzalez, M. A., Adams, D. J., Ng, B. L., Petty, R., Venkitaraman, A. R., Arends, M. J. and Bradley, A. (2005). The *RASSF1A* isoform of *RASSF1* promotes microtubule stability and suppresses tumorigenesis. *Mol. Cell. Biol.* **25**, 8356-8367.

van Engeland, M., Roemen, G. M., Brink, M., Pachen, M. M., Weijenberg, M. P., de Bruine, A. P., Arends, J. W., van den Brandt, P. A., de Goeij, A. F. and Herman, J. G. (2002). K-ras mutations and RASSF1A promoter methylation in colorectal cancer. *Oncogene* **21**, 3792-3795.

Vavvas, D., Li, X., Avruch, J. and Zhang, X. F. (1998). Identification of Nore1 as a potential Ras effector. *J. Biol. Chem.* **273**, 5439-5442.

Vos, M. D., Ellis, C. A., Bell, A., Birrer, M. J. and Clark, G. J. (2000). Ras uses the novel tumor RASSF1 as an effector to mediate apoptosis. *J. Biol. Chem.* **275**, 35669-35672.

Vos, M. D., Ellis, C. A., Elam, C., Ulku, A. S., Taylor, B. J. and Clark, G. J. (2003a).

RASSF2 is a novel K-Ras-specific effector and potential tumor suppressor. *J. Biol. Chem.* **278**, 28045-28051.

Vos, M. D., Martinez, A., Ellis, C. A., Vallecorsa, T. and Clark, G. J. (2003b). The pro-apoptotic Ras effector Nore1 may serve as a Ras-regulated tumor suppressor in the lung. *J. Biol. Chem.* **278**, 21938-21943.

Vos, M. D., Martinez, A., Elam, C., Dallol, A., Taylor, B. J., Latif, F. and Clark, G. J. (2004). A role for the RASSF1A tumor suppressor in the regulation of tubulin polymerization and genomic stability. *Cancer Res.* **64**, 4244-4250.

Vos, M. D., Dallol, A., Eckfeld, K., Allen, N. P. C., Donninger, H., Hesson, L. B., Calvisi, D., Latif, F. and Clark, G. J. (2006). The RASSF1A tumor suppressor activates Bax via MOAP-1. *J. Biol. Chem.* **281**, 4557-4563.

Wagner, K. J., Cooper, W. N., Grundy, R. G., Caldwell, G., Jones, C., Wadey, R. B., Morton, D., Schofield, P. N., Reik, W., Latif, F. et al. (2002). Frequent RASSF1A tumor suppressor gene promoter methylation in Wilms' tumour and colorectal cancer. *Oncogene* **21**, 7277-7282.

Wassmann, K. and Benezra, R. (2001). Mitotic checkpoints: from yeast to cancer. *Curr. Opin. Genet. Dev.* **11**, 83-90.

Whang, Y. M., Kim, Y. H., Kim, J. S. and Yoo, Y. D. (2005). RASSF1A suppresses the c-Jun-NH_2-kinase pathway and inhibits cell cycle progression. *Cancer Res.* **65**, 3682-3690.

Wong, M. L., Tao, Q., Fu, L., Wong, K. Y., Qiu, G. H., Law, F. B., Tin, P. C., Cheung, W. L., Lee, P. Y., Tang, J. C. et al. (2006). Aberrant promoter hypermethylation and silencing of the critical 3p21 tumour suppressor gene, RASSF1A, in Chinese oesophageal squamous cell carcinoma. *Int. J. Oncol.* **28**, 767-773.

Yabuta, N., Fujii, T., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Nishiguchi, H., Endo, Y., Toji, S., Tanaka, H., Nishimune, Y. et al. (2000). Structure, expression, and chromosome mapping of LATS2, a mammalian homologue of the Drosophila tumor suppressor gene lats/warts. *Genomics* **63**, 263-270.

Yeo, W., Wong, W. L., Wong, N., Law, B. K., Tse, G. M. and Zhong, S. (2005). High frequency of promoter hypermethylation of RASSF1A in tumorous and non-tumourous tissue of breast cancer. *Pathology* **37**, 125-130.

Yoon, J.-H., Dammann, R. and Pfeifer, G. P. (2001). Hypermethylation of the CpG island of the RASSF1A gene in ovarian and renal cell carcinomas. *Int. J. Cancer* **94**, 212-217.

Yu, H. (2007). Cdc20: a WD40 activator for a cell cycle degradation machine. *Mol. Cell* **27**, 3-16.

Yu, M. Y., Tong, J. H., Chan, P. K., Lee, T. L., Chan, M. W., Chan, A. W., Lo, K. W. and To, K. F. (2003). Hypermethylation of the tumor suppressor gene RASSF1A and frequent concomitant loss of heterozygosity at 3p21 in cervical cancers. *Int. J. Cancer* **105**, 204-209.

Zachariae, W. and Nasmyth, K. (1999). Whose end is destruction: cell division and the anaphase-promoting complex. *Genes Dev.* **13**, 2039-2058.