EUROPEAN JOURNAL OF CANCER 46 (2010) 2986–2995

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Protein kinase A-mediated phosphorylation of the RASSF1A tumour suppressor at Serine 203 and regulation of RASSF1A function

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ARTICLE INFO

Article history:
Received 8 February 2010
Received in revised form 20 June 2010
Accepted 23 June 2010
Available online 23 July 2010

Keywords:
RASSF1A
Tumour suppressor, PKA
Phosphorylation
Apoptosis

ABSTRACT

Epigenetic inactivation of the *Ras-Association Domain Family 1A* (*RASSF1A*) gene is one of the most frequent alterations detected in cancer. The tumour suppressor function of RASSF1A contributes to cell cycle progression, microtubule stabilisation and apoptotic signalling. Here we investigated the putative phosphorylation sites of RASSF1A and the functional consequences. RASSF1A is mainly phosphorylated at Serine 203 within its Ras association domain. Phosphorylation at this site is accomplished by protein kinase A (PKA) and is reduced and elevated by PKA-specific inhibitors and activators, respectively. Functionally, an alanine substitution of Serine 203 (S203A) slightly affected the microtubule stability mediated by RASSF1A ($p < 0.05$). Interestingly, the inhibition of PKA and the S203A substitution of RASSF1A resulted in a reduced rate of apoptotic cells induced by RASSF1A. Moreover, RASSF1A-mediated upregulation of *p21* and *BAX* was observed. This induction was reduced when the S203A substitution was present or when PKA activity was inhibited. In summary our data show that RASSF1A is phosphorylated by PKA and this phosphorylation may affect apoptotic signalling of RASSF1A. Thus epigenetic silencing of RASSF1A may counteract its proapoptotic function in cancer.

1. Introduction

The Ras-Association Domain Family 1 (RASSF1) was identified using a yeast-two-hybrid screen through its interaction with XPA, a protein known to function in DNA excision repair.[1] The C-terminus of RASSF1 displayed a high homology to mouse Nore1 (also termed Rassf5), a known Ras effector.[2] We have further identified two main transcripts: *RASSF1A* and *RASSF1C*. Both variants share four common exons, encoding a Ras-Association (RalGDS/AF-6) domain, from which the family name was derived.[3] The *RASSF1* gene is located on the small arm of chromosome 3 (3p21.3).[1] Earlier studies on the loss of heterozygosity (LOH) were investigating the very same chromosomal region in various tumour entities and had already proposed the existence of tumour suppressor genes at 3p21.3.[4,5] Subsequently, we showed that RASSF1A acts as a tumour suppressor and is frequently inactivated in human cancers.[1] RASSF1A is implicated in various cellular mechanisms including apoptosis, cell cycle control and microtubule stabilisation, though little is known about the underlying mechanisms.[6]

The N-terminus of RASSF1A has high homology to a cysteine-rich diacylglycerol/phorbol ester-binding (DAG) domain,

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0959-8049/$ - see front matter
doi:10.1016/j.ejca.2010.06.128

also known as the protein kinase C conserved region 1 (C1), which contains a central zinc finger.[7] The amino acid sequence W125 to K138 of RASSF1A matches a putative ataxia telangiectasia-mutated (ATM) kinase phosphorylation consensus motif and S131 is effectively phosphorylated by ATM.[8,9] In the C-terminal part of RASSF1A and RASSF1C a novel protein–protein interaction domain called SARAH (Sav-RASSF-Hpo) has been predicted by *in silico* analysis.[10] In Drosophila, Salvador (Sav, the human homologue is named WW45) acts as a scaffold protein that interacts with the proapoptotic kinase Hippo (Hpo, human homologue MST).[11–13] Hpo is able to phosphorylate the kinase Warts (human homologue LATS), which in Drosophila leads to cell cycle arrest and apoptosis.[12–14] Praskova et al. previously showed that human RASSF1A interacts with MST1 through the C-terminus.[15]

It was shown that RASSF1A is being phosphorylated within its RA domain (position 202/203) by Aurora A during mitosis, and it was reported that this interfered with the ability of RASSF1A to interact with microtubules and abolished its ability to induce M-phase cell cycle arrest.[16,17] It was suggested that RASSF1A plays a role in the activation of Aurora A, and it was proposed that RASSF1A may function as a scaffold for Aurora A and its actual activator.[18] Aurora B-mediated phosphorylation of RASSF1A was also reported and has a critical role in regulating cytokinesis.[19] Verma et al. showed that RASSF1A is a substrate for protein kinase C (PKC) and that the tumour suppressor becomes phosphorylated by the kinase at positions S197 and S203.[20]

Here we aimed to reveal novel phosphorylation sites of RASSF1A and we report that position S203 of RASSF1A is also phosphorylated by protein kinase A (PKA). This residue is involved in microtubule stability and induction of apoptosis mediated by RASSF1A.

2. Materials and methods

2.1. Plasmids

RASSF1A was cloned in vector pMAL-c2 (New England Biolabs, Frankfurt, Germany). Mutated MBP-RASSF1A constructs were made with the QuickChange XL Mutagenesis Kit (Stratagene, Amsterdam, Netherlands) and specific primers. MBP-RASSF1A-ΔATM (deletion of aas 125–138 of RASSF1A) was generated with primer 5′-CGTGGACGAGCCTGTGGAGATCAAGGAG TACAATGC and the complementary primer. For MBP-RASSF1A-C1 (C1) the aas 1–121 were cloned in pMAL-c2.

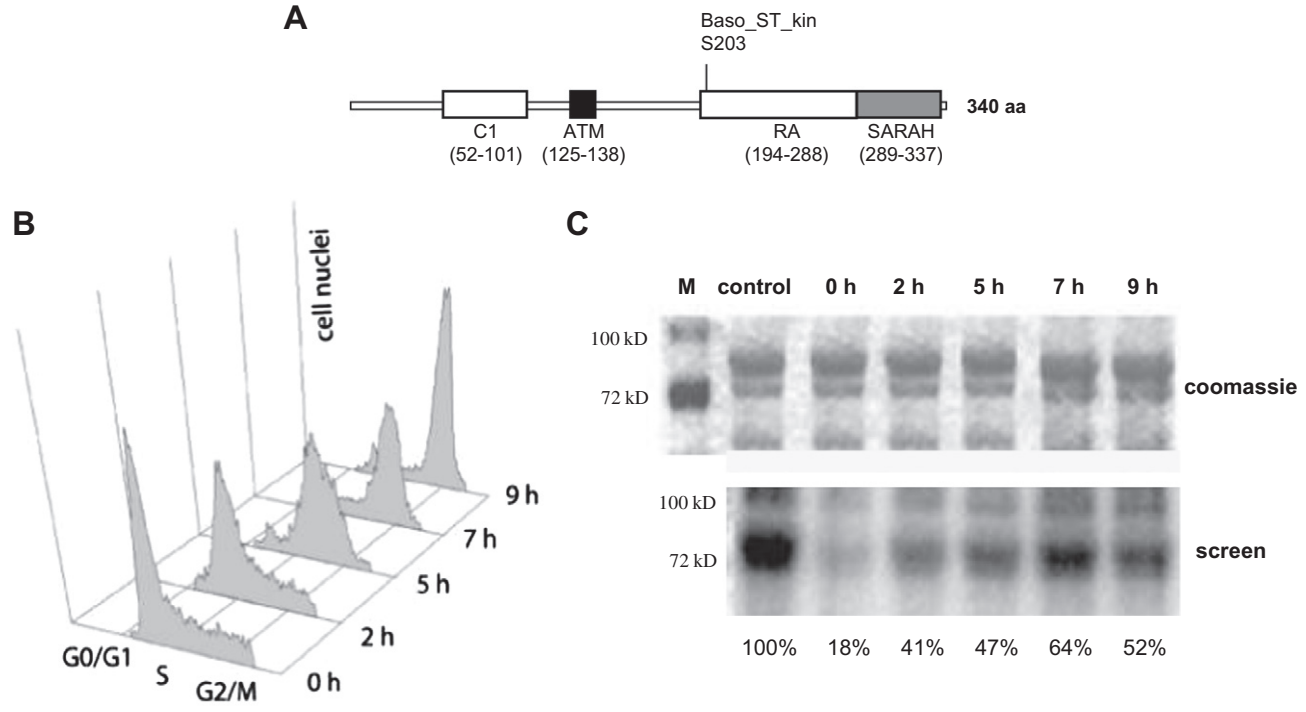

Fig. 1 – RASSF1A phosphorylation. (A) In *silico* analysis of RASSF1A phosphorylation sites. Protein sequence of RASSF1A (340aa) was analysed with Motif Scan (Scansite 2.0) with high stringency. A Basophilic serine/threonine kinase group (Baso_ST_kin) at Serine 203 (S203) was predicted. The protein kinase C1 domain (C1), the putative ataxia telangiectasia mutated kinase substrate (ATM), the Ras association domain (RA) and the Sav-RASSF-Hpo domain (SARAH) of RASSF1A with corresponding amino acid positions are indicated. (B) IGR1 cells were arrested with methotrexate in G1. After cell cycle release, nuclei were stained with propidium iodide and the DNA content was analysed by flow cytometry at the indicated time points. (C) Phosphorylation of RASSF1A. IGR1 protein extracts from different cell cycle stages (0 h, 2 h, 5 h, 7 h and 9 h) and 50 μCi γ^{32}P-ATP were added to precipitated RASSF1A-MBP. The protein extract from unsynchronised IGR1 cells was utilised as control. Labelled RASSF1A-MBP were separated on a 10% SDS–polyacrylamide gel and stained with coomassie and exposed to a screen. Relative phosphorylation of RASSF1A was calculated from two independent experiments and is indicated (control = 100%). The sizes of a protein ladder (M) are indicated.

Substitutions at S203F, S203A, S131F, A133S and S131F/A133S were generated with primers: 5′-GTCAGGCGCCGCACTTTCTTTTACCTGCCC; 5′-GTCAGGCGCCGCACTGCATTTTACCTGCCCAAGG, 5′-GGGAGACACCTGACCTTTTTCAAGCTGAGATTGAGC, 5′-GGAGACACCTGACCTTTCTCAATCTGAGATTG, 5′-GGGAGACACCTGACCTTGCCCAATCTGAGATTGAGCAGAAGA, respectively, together with the corresponding complementary primer (mutated codons are underlined). All constructs were verified by sequencing (Seqlab, Göttingen, Germany).

2.2. Cell culture and protein extract

Melanoma cell line IGR1 (DSMZ, Braunschweig, Germany) was cultured in DMEM and 10% FCS in a humidified atmosphere with 5% CO_2 at 37 °C. IGR1 cells were washed twice with PBS and incubated in cold RIPA buffer and protease inhibitors (Roche, Heidelberg, Germany) for 10 min. Cells were disrupted with a needle and centrifuged at 14,000 rpm for 10 min. The protein concentration in the supernatant was determined and 200 μg of protein extract was used for each phosphorylation reaction.

2.3. Phosphorylation assays of RASSF1A

MBP fusion proteins were expressed in *Escherichia coli* TB1. Cells were cultured in LB-medium and 100 μg/l ampicillin at 37 °C. At OD 0.6 cells were induced for 4 h with 0.5 mM IPTG. To purify proteins, cells were harvested and resuspended in amylose resin buffer (20 mM Tris, 200 mM NaCl and 1 mM EDTA) and sonicated on ice. The cell debris was centrifuged and the supernatant was aliquoted and frozen at –80 °C. Eighty microlitres of amylose resin (NEB, Frankfurt,

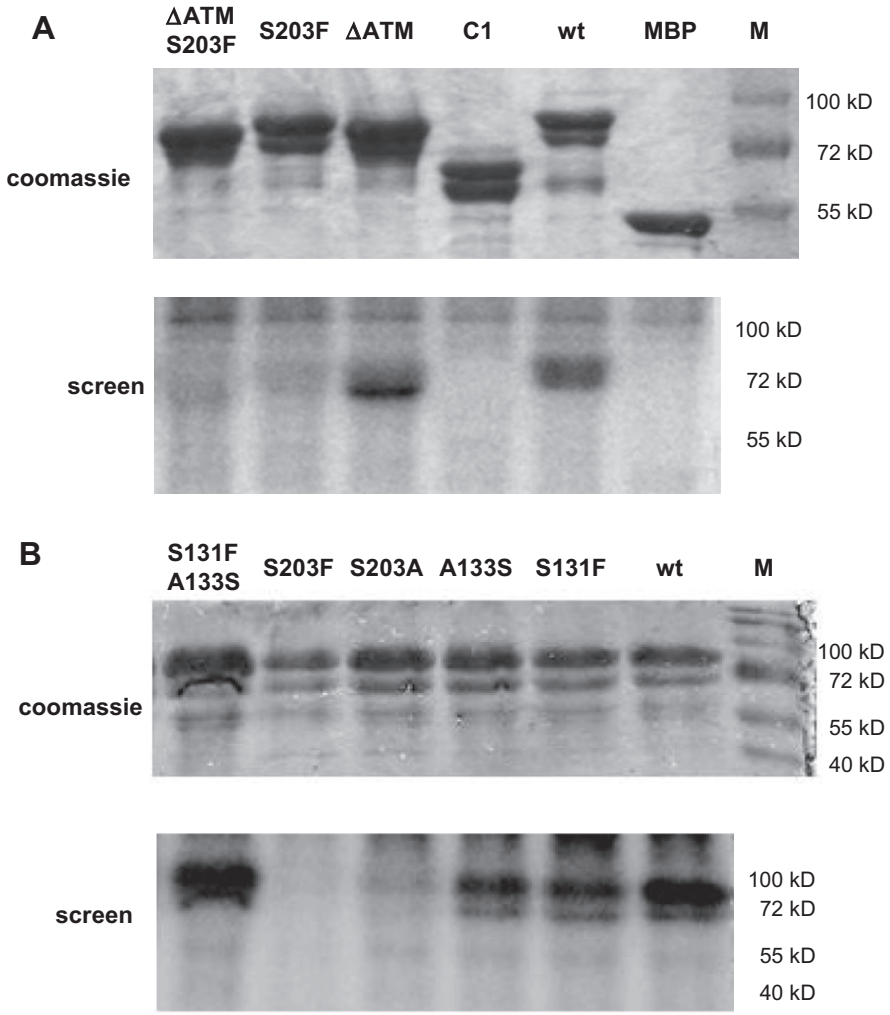

Fig. 2 – Phosphorylation of RASSF1A occurs preferentially at codon 203. (A) Maltose-binding protein (MBP), MBP-RASSF1A protein (wt), RASSF1A with a deletion of the putative ATM-kinase phosphorylation site (ΔATM), protein kinase C1 domain (C1) of RASSF1A and a substitution of RASSF1A at Serine 203 (S203F) were purified and incubated with IGR1 protein extract and γ^{32}P-ATP. Two to 5 μg of protein were dissolved on a 10% polyacrylamide gel, stained with coomassie and exposed on a screen. The sizes of a protein ladder (M) are indicated. (B) MBP-RASSF1A (wt) and mutated forms with substitutions at codon 131 (S > F), codon 133 (A > S) and codon 203 (S > F or S > A) were expressed in *E. coli* and purified. Two to 5 μg of protein were incubated with IGR1 protein extract and γ^{32}P-ATP. Proteins were separated and stained with coomassie and exposed on a phosphor screen.

Germany) were added to 100 μl *E. coli* cell extract with appropriated constructs and incubated for 1 h at 4 °C. Amylose resin was washed twice and incubated with 200 μg of IGR1 protein extract at 4 °C for 1 h. The resins were resuspended in 100 μl kinase buffer with 50 μCi γ^{32}P-ATP and incubated at 30 °C for 30 min. Subsequently, the amylose resins were washed twice with buffer and resuspended in 20 μl protein loading buffer, denatured and resolved by SDS–PAGE and stained with coomassie. The gel was analysed on phosphor image scanner.

2.4. *Protein kinases, PKA activity assay and inhibitors*

To investigate kinases, which phosphorylate RASSF1A, the following enzymes were obtained: Cdc2 protein kinase (NEB, Frankfurt, Germany), protein kinase B (PKB/Akt1, NEB, Frankfurt, Germany), casein kinase I (CKI, NEB, Frankfurt, Germany) and protein kinase A (PKA, NEB, Frankfurt, Germany). For the reactions 20 U of PKB/Akt1, 20 U of Cdc2, 1000 U of CKI and 1250 U of PKA were added together with 50 μCi γ^{32}P-ATP to MBP-RASSF1A at 30–37 °C for 90 min in supplied reaction

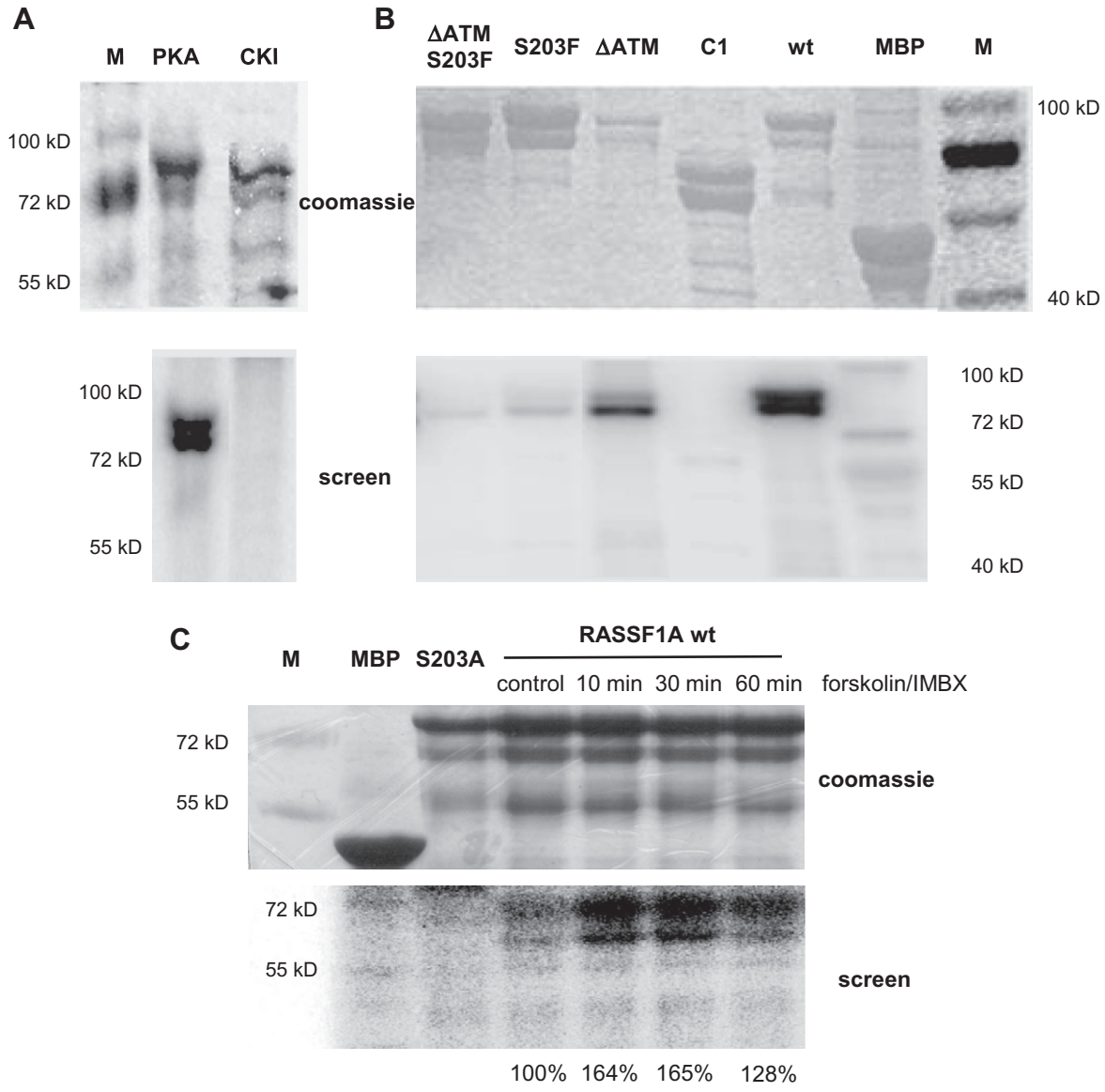

Fig. 3 – Phosphorylation of RASSF1A by protein kinase A (PKA). (A) RASSF1A-MBP was purified and incubated with PKA and casein kinase I (CKI) and γ^{32}P-ATP. Two to 5 μg of protein were dissolved on a 10% polyacrylamide gel, stained with coomassie and exposed on a screen. The sizes of a protein ladder (M) are indicated. (B) Maltose-binding protein (MBP), RASSF1A-MBP (wt), RASSF1A-MBP with a deletion of the putative ATM-kinase phosphorylation site (ΔATM, the protein kinase C1 domain (C1) of RASSF1A-MBP and a substitution of RASSF1A at Serine 203 (S203F) and RASSF1A-ΔATM-S203F-MBP (ΔATM/S203F) were purified and incubated with PKA and γ^{32}P-ATP and dissolved. (C) The indicated proteins were incubated with 400 μg of IGR1 extract and 40 μCi γ^{32}P-ATP in the presence of 20 μM forskolin and 500 μM IBMX for 10 to 60 min. Labelled proteins were dissolved and analysed. Phosphorylation of RASSF1A was quantified relative to MBP (0%) and mock control (100%).

buffers. The products were resolved by SDS–PAGE and exposed to a phosphor screen.

Activity of PKA was determined through the SignaTECT cAMP-dependent Protein Kinase assay (Promega, Mannheim Germany). Phosphorylation reactions were performed according to the manufacturer's instructions with 10 μCi γ^{32}P-ATP and biotinylated Kemptide (LRRASLG) in the reaction. Briefly, each reaction was performed by using 15 μg protein extract or 1250 U of PKA and spotted on biotin capture membranes. Phosphorylated substrate was quantified on a phosphor screen.

The following kinase inhibitors and activators were utilised: wortmannin (Biomol, Hamburg, Germany), staurosporine (Sigma–Aldrich, Taufkirchen, Germany), H89 (Sigma–Aldrich, Taufkirchen, Germany), aloisine A (Merck, Darmstadt, Germany), forskolin (Sigma–Aldrich, Taufkirchen, Germany) and IBMX (Sigma–Aldrich, Taufkirchen, Germany).

2.5. *RASSF1A phosphorylation by PKACα*

CDNA of PKACα (IRAKp961P0684Q) was obtained from RZPD (German Resource Center for Genome Research, Berlin, Germany). PKACα, MST1, CREB and RASSF1A constructs were cloned in pCMV-Tag1 (Stratagene, Amsterdam, Netherlands). FLAG-tagged constructs were transfected with Lipofectamin 2000 (Invitrogen, Carlsbad, USA) in HEK293 cells. Cells were cultured for 24 h and total protein was extracted in FLAG lysis buffer. FLAG-tagged proteins were precipitated with anti-FLAG M2 affinity gel (Sigma–Aldrich, Taufkirchen, Germany) and incubated for 90 min in 20 μl PKA kinase assay buffer containing 10 μCi γ^{32}P-ATP at 30 °C. Samples were separated on a 10% SDS–PAGE and transferred onto a PVDF membrane. Proteins were visualised with anti-FLAG M2 antibody (Sigma–Aldrich, Taufkirchen, Germany) and BCIP/NBT. Membranes were exposed to phosphor screens.

2.6. *Apoptotic assays*

A549 cells were seeded on glass slides and transfected with different RASSF1A constructs (EYFP; EGFP), using HEKfectin (BioRad; München; Deutschland), and analysed after 4 d of incubation. Thereafter cells were fixed with paraformaldehyde, permeabilised with Triton X, DAPI stained and embedded in MOWIOL for fluorescent analysis. Transfected cells ($n > 100$) were analysed for intact, condensed, fragmented or missing nucleus. TUNEL staining was used to confirm

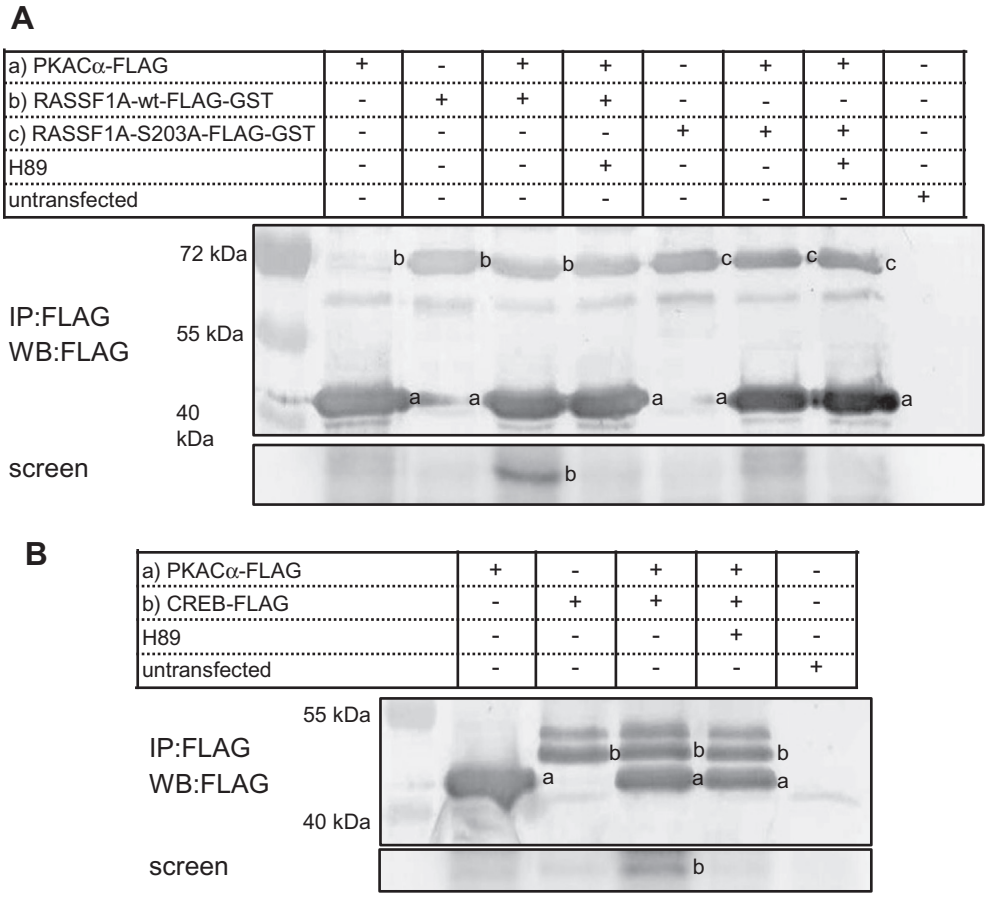

Fig. 4 – PKA-mediated RASSF1A phosphorylation. (A) FLAG-tagged PKACα and RASSF1A variants (wt and S203A) were transfected in HEK293T cells and bound to anti-FLAG-antibodies and incubated with γ^{32}P-ATP. 10 μM of the PKA inhibitor H89 was applied. Purified proteins were dissolved on a 10% SDS–polyacrylamide gel and analysed by Western blot with anti-FLAG-antibodies. Membrane was exposed to a phosphor screen. (B) FLAG-tagged PKACα and CREB were co-transfected in HEK293 cells and bound to anti-FLAG-antibodies and incubated with γ^{32}P-ATP and analysed as described above. Sizes of a protein ladder are indicated.

fragmented nuclei to be apoptotic using the In Situ Cell Death Detection Kit; TMR red (Roche; Mannheim; Deutschland).

2.7. Microtubule stability

A549 cells were seeded on glass slides and transfected with different RASSF1A constructs (EYFP; EGFP), using HEKfectin (BioRad; München; Deutschland): Microtubule stability was analysed after 1 d of incubation. Thereafter cells were fixed with paraformaldehyde, permeabilised with Triton X and incubated for 4 h with nocodazole (5 ng/μl) (Sigma–Aldrich, Taufkirchen, Germany). Slides were then incubated consecutively with anti-α-tubulin (A11126 Invitrogen; Carlsbad, USA) and Alexa Fluor 568 (A11004 Molecular Probes; Carlsbad; USA), then stained with DAPI and embedded in MOWIOL for fluorescent analysis. Transfected cells ($n > 250$) were analysed for positive or negative α-tubulin stain.

2.8. Expressional analysis of RASSF1A-target genes

A549 cells were transfected using HEKfectin (BioRad; München; Deutschland) with FLAG-constructs of CMV-empty vector, RASSF1A and mutants. RNA was isolated after 4 d with TRIzol reagent (Invitrogen; Karlsruhe; Deutschland), DNase treated (Fermentas; St. Leon-Rot; Deutschland; Promega; Mannheim; Deutschland) and reversely transcribed using M-MLV (Promega; Mannheim; Deutschland). Realtime PCR was performed using 100 ng of cDNA with primers for *p21*: 5′-CCTTGTGCCTCGGTCAGGGGAG3′; 5′-GGCCCTCGCGCTTCC-AGGAC3′ and *BAX*: 5′-AACTGGGGCCGGGTTGTCGC3′; 5′-CGC-GGTGGTGGGGGTGAGG3′ and *PUMA*: 5′-GCGGCGGATGGCG-GACGA; 5′-CTGACGTCCACCGGGCGGGT.

3. Results

3.1. Phosphorylation of RASSF1A at codon 203

In previous studies phosphorylation of RASSF1A at Serine 203 by aurora kinases and PKC was reported.[16,19,20] Additional putative phosphorylation site (e.g. Serine 131 by ATM kinase) was previously reported[8] and recently confirmed.[9] To reveal novel phosphorylation sites of RASSF1A we analysed its protein sequence *in silico* by Scansite 2.0.[21] Only serine at codon 203 was identified as a putative phosphorylation site for basophilic serine/threonine kinases (Fig. 1A). Additionally we analysed the phosphorylation and expression of RASSF1A during

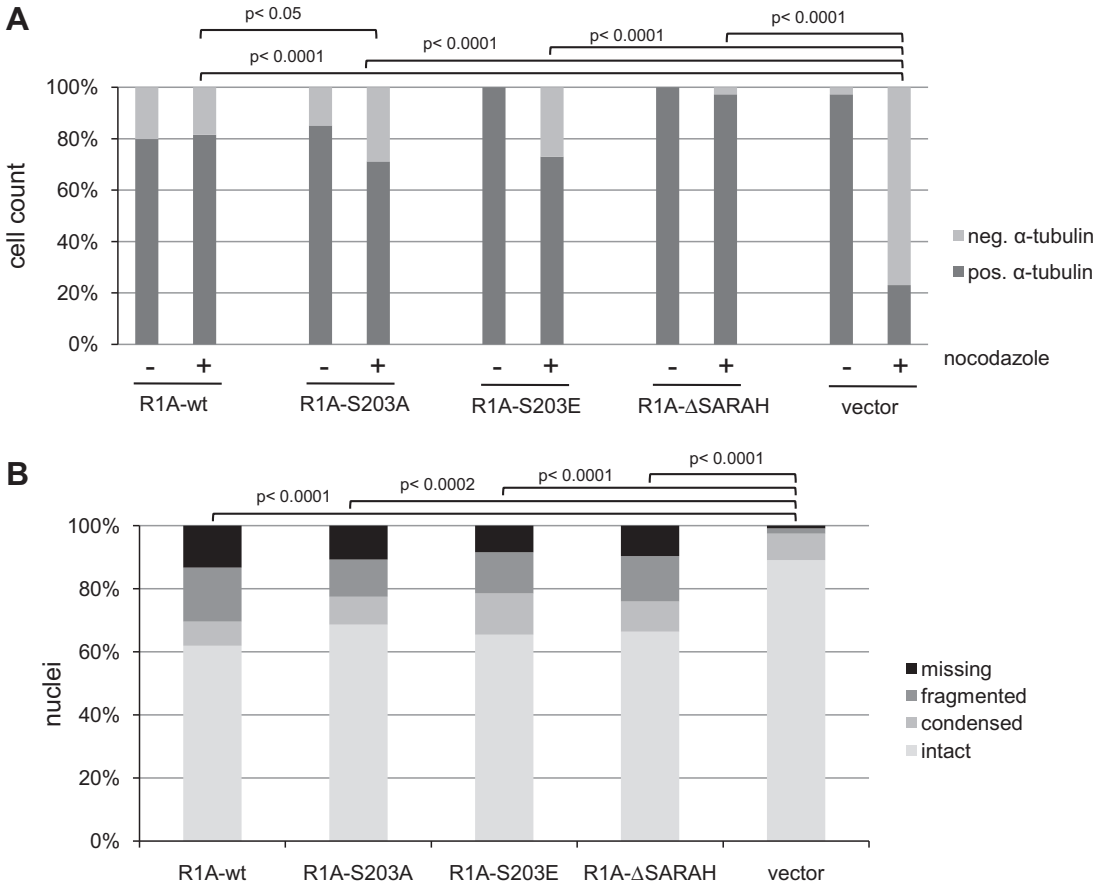

Fig. 5 – RASSF1A-induced microtubule stability (A) and apoptosis (B). (A) A549 cells were transfected with the indicated fluorescent RASSF1A (R1A) constructs and control vector (pEGFP). Depolymerisation of microtubule was induced with 5 ng/μl nocodazole. Cells were fixed and stained with DAPI and anti-α-tubulin. Fluorescent cells (n>250) were counted. The percentage of α-tubulin positive cells was calculated and significant results are indicated. (B) RASSF1A-induced apoptosis. A549 cells were transfected and after 4 d cells were fixed and stained with DAPI. Fluorescent cells ($n > 100$) were counted and the percentage of intact, condensed, fragmented and missing nuclei was determined.

the cell cycle (Fig. 1 and Supplementary Fig. 1). Therefore we synchronised IGR1 cells in G1 and cell cycle progression was determined (Fig. 1B). Cell cycle-dependent expression of RASSF1A was not observed (Supplementary Fig. 1), however a cell cycle-dependent phosphorylation of RASSF1A was found (Fig. 1C). Phosphorylation of RASSF1A during mitosis was already reported.[16] However, we also observed significant phosphorylation of RASSF1A in G1 phase (Fig. 1C). Interestingly, the disappearance of both aurora kinases in G1 has been shown.[22] To determine which residues of RASSF1A are phosphorylated in G1 phase, we created different truncated and mutated versions of RASSF1A (Fig. 2). Neither deletion of the ATM phosphorylation site nor the substitution of S131 with phenylalanine significantly reduced the phosphorylation rate of RASSF1A (Fig. 2A and B). However, substitutions at Serine 203 with phenylalanine or alanine strongly diminished the phosphorylation of RASSF1A (Fig. 2). The N-terminal fragment containing amino acids 1–121 was not phosphorylated (Fig. 2A). Moreover, we tested a common polymorphism at codon 133 (A > S), which was correlated with an increased risk for tumour incidence.[23,24] Substitution of codon 133 did not change the phosphorylation of RASSF1A compared to wt-protein (Fig. 2B).

3.2. *Phosphorylation of RASSF1A by PKA*

To identify novel basophilic serine/threonine kinases that phosphorylate RASSSF1A at Serine 203, we tested different kinase inhibitors (Supplementary Fig. 2). Staurosporine was the only inhibitor, which reduced RASSF1A phosphorylation at less than 100 nM. Staurosporine is able to inhibit several serine/threonine kinases including PKC and PKA.[25] Using a PKA phosphorylation system we compared the capability of staurosporine to inhibit the phosphorylation of *bona fide* PKA substrate (Kemptide) through IGR1 protein extract or recombinant PKA (Supplementary Fig. 2B and C). Inhibition of IGR1 extract was similar for RASSF1A and Kemptide. Subsequently the ability of recombinant PKA to phosphorylate RASSF1A was investigated (Fig. 3A and B). PKA was able to phosphorylate RASSF1A at Serine 203, however Cdc2, PKB/Akt1 and casein kinase I (CKI) were not able to phosphorylate RASSF1A (Fig. 3A and data not shown). Additionally, induction of PKA activity by forskolin and IBMX in IGR1 extract enhanced phosphorylation of RASSF1A almost twice (Fig. 3C). Moreover, *in vivo* phosphorylation of RASSF1A by overexpressing PKACα and different RASSF1A variants was analysed (Fig. 4). FLAG-tagged PKACα effectively phosphorylated RASSF1A and CREB (Fig. 4A and B). This reaction was inhibited by H89 a potent inhibitor of PKA and by substitution of Serine 203 through alanine, but not by A133S (Fig. 4 and Supplementary Fig. 3). Phosphorylation of RASSF1A by PKACγ was also observed (data not shown). Our data suggest that serine at codon 203 is a PKA-mediated phosphorylation site of RASSF1A.

3.3. *Functional relevance of Serine 203 of RASSF1A*

We analysed the functional relevance of RASSF1A S203A substitution including microtubule stability and apoptosis.

a) MST1-GST-FLAG	+		+		+		+	-	+
b) RASSF1A-FLAG	+		-		-		-	-	-
c) RASSF1A-S203A-FLAG	-		+		-		-	-	-
d) RASSF1A-S203E-FLAG	-		-		+		-	-	-
e) RASSF1A-A133S-FLAG	-		-		-		+	-	-
f) RASSF1A-ΔSARAH-FLAG	-		-		-		-	-	+
untransfected	-		-		-		-	+	-

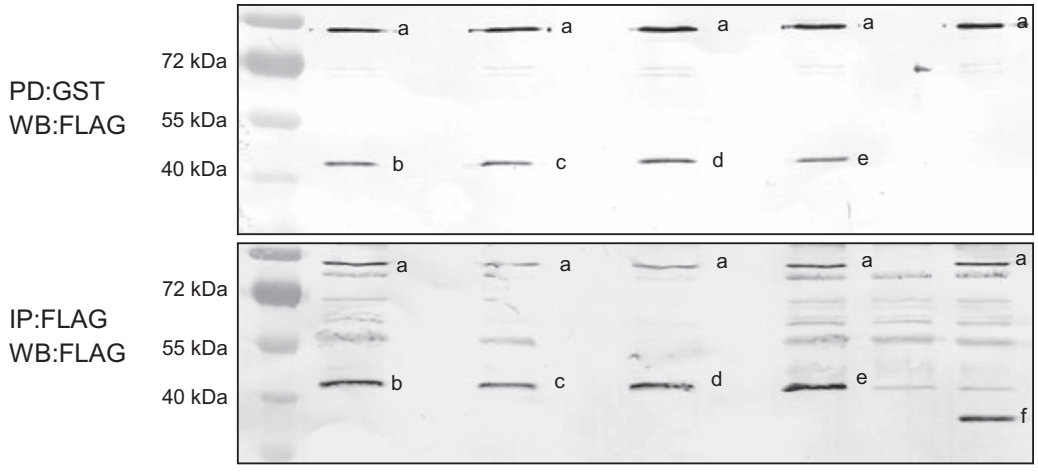

Fig. 6 – RASSF1A binding to MST1. Constructs that express MST1-GST-FLAG and indicate RASSF1A-FLAG variants were co transfected into HEK293 cells. After 2 d, total protein was extracted and GST-tagged proteins were pulled down (PD) with glutathione sepharose or immunoprecipitated (IP) with anti-FLAG-agarose. Samples were separated on a 10% PAGE gel and blotted. The precipitated and co-precipitated proteins were determined with anti-FLAG-antibodies (FLAG-blot). Sizes of a protein marker are indicated.

Therefore we generated fluorescent-tagged variants of RASSF1A that harboured substitutions at codon 203 (S203A and S203E) and truncation of the SARAH domain (ΔSARAH). These forms were transfected in A549 lung cancer cells and their localisation with α-tubulin was analysed (Supplementary Fig. 4). Subsequently, the ability of RASSF1A and its variants to stabilise microtubules under nocodazole treatment was determined (Fig. 5A). All the analysed RASSF1A forms significantly counteracted microtubule depolymerisation, however, substitutions at codon 203 were less effective.

Additionally, we analysed the RASSF1A-induced apoptosis in A549 cells. Transfection of fluorescent RASSF1A caused apoptosis indicated by nuclei condensation/fragmentation and DNA breaks (Supplementary Fig. 5). Compared to fluorescent controls (EGFP and EYFP) all variants of RASSF1A (S203A, S203E and ΔSARAH) significantly induced missing, fragmented and condensed nuclei (Fig. 5B). Interestingly, RASSF1A S203A exhibited a reduced rate of apoptotic cells (Fig. 5B). To test if substitutions of codon 203 interfered with the ability of RASSF1A to bind the proapoptotic kinase MST1, we performed pull down assays with different tagged forms of RASSF1A (Fig. 6). Only deletion of the SARAH protein interaction domain abolished the binding of MST1 to RASSF1A. It has been reported that the RASSF1A-induced apoptosis is accompanied by activation of the transcription factor p73 through its cofactor YAP1 and upregulation of the p73-target genes.[26] Therefore we analysed the RASSF1A-induced expression of *p21*, *BAX* and *PUMA* (Fig. 7A). Transfection of wildtype RASSF1A increased the expression of *p21* and *BAX*, but not of *PUMA* and *Caspase 3* (data not shown). Interestingly we observed that RASSF1A S203A and the PKA inhibitor H89 reduced the induction rate of *p21* and *BAX* (Fig. 7A) and also significantly decreased the amount of apoptotic cells (Fig. 7B). In summary these data suggest that PKA-mediated phosphorylation of RASSF1A is involved in its proapoptotic function.

4. Discussion

Modification of tumour suppressors by phosphorylation is the most general control mechanism of cells to regulate protein activity and signal transduction. Here we found that the cell extract isolated form IGR1 cells phosphorylated RASSF1A in a cell cycle-dependent manner. We and others have already reported that RASSF1A binds the mitotic kinase Aurora A and that RASSF1A is phosphorylated in mitosis at Serine 203.[16–18] RASSF1A may function as a scaffold to bring together Aurora A and its activators.[18] Phosphorylation of RASSF1A

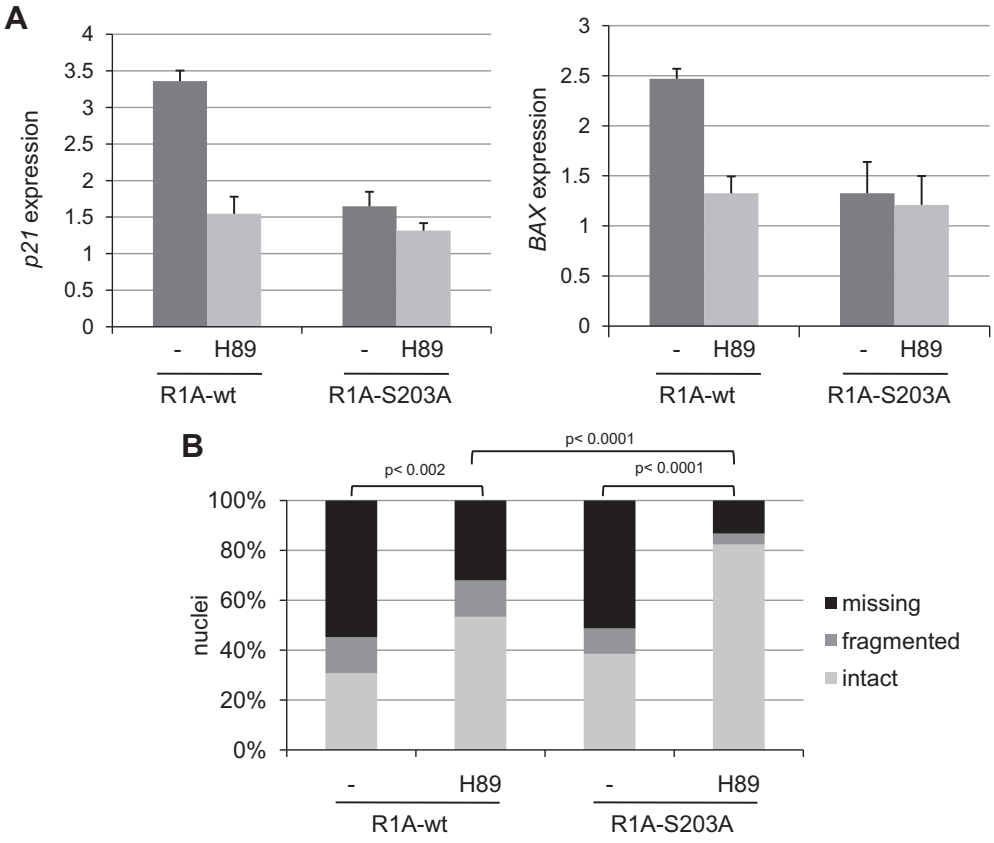

Fig. 7 – RASSF1A-mediated induction of *p21* and *BAX* or apoptosis. (A) A549 cells were transfected with different RASSF1A-FLAG constructs (R1A) and control vector (pCMV-Tag1). Cells were treated with 50 μM H89 and compared to untreated controls (–). RNA was isolated after 4 d and expression of *p21* and *BAX* was analysed by qRT-PCR. Relative expression was normalised to β-*actin* and vector control (= 1). Means and standard deviations are indicated. (B) RASSF1A-induced apoptosis. A549 cells were transfected and after 4 d cells were fixed and stained with DAPI. Fluorescent cells ($n > 100$) were counted and the percentage of intact, condensed, fragmented and missing nuclei was determined. Significant results are indicated.

during late mitosis by Aurora B was also reported and has a critical role in regulating cytokinesis.[19] Both Aurora kinases phosphorylate RASSF1A at Serine 203, however phosphorylation at Threonine 202 by Aurora A was also reported.[16,17,19] In addition to the cell cycle dependence, a significant phosphorylation capability was also present in the IGR1 extract isolated at G1 phase. It has been shown that both Aurora kinases are absent in G1.[22] Therefore we investigated other basophilic serine/threonine kinases that phosphorylate RASSF1A during interphase and identified protein kinase A (PKA) as a novel RASSF1A-specific kinase. The phosphorylation site of RASSF1A by PKA was also Serine 203. In contrast to the Aurora kinases, which bind RASSF1A physically, we found no direct binding of RASSF1A to PKA (data not shown).

PKA is involved in the phosphorylation of several tumour suppressor genes, including the LKB1, NF1, NF2 and Wilms tumour 1.[27–30] For WT1 it was reported that both PKA and PKC phosphorylate it.[30] Phosphorylation of RASSF1A by PKC was also reported.[20] Verma et al. show that Serine 197 and 203 are phosphorylated *in vitro* and *in vivo* by PKC and substitutions at these positions altered the microtubule stability associated with RASSF1A. We also found that position 203 significantly affected RASSF1A-mediated microtubule stability (Fig. 5A). Previously, we have mapped the domain, which is required for microtubule association and stabilisation to the region from amino acid 120–288.[31] The SARAH domain is not part of this domain and therefore we observed no such effect when deleting this fragment (Fig. 5).

The SARAH domain is responsible for binding the proapoptotic kinases MST1 and MST2.[15] It was proposed that RASSF1A induces apoptosis via binding to MST2, which is released from inhibition by RAF-1 following Fas activation.[26] Subsequently MST2, activated by the interaction with RASSF1A, binds to LATS, and YAP1 becomes phosphorylated by LATS and dissociates from LATS (Fig. 8). YAP1 then translocates to the nucleus and forms a complex with p73 ultimately leading to the transcription of *PUMA*, which initiates apoptosis.[26] Other p73 target genes include *BAX* and *p21*.[32,33] We have previously reported that reexpression of RASSF1A in A549 cells induces p21 expression.[34] Here we were not able to detect increased expression of *PUMA*, however we found RASSF1A-induced expression of *BAX* and *p21*. This indicates that RASSF1A-mediated tumour suppression function also involves the activation of these two gene products (Fig. 8). This effect and apoptosis are reduced when a substitution at amino acid 203 is present. Moreover, inhibition of PKA by H89 reduced RASSF1A-mediated apoptosis significantly (Fig. 7). This suggests that phosphorylation of Serine 203 also contributes to the apoptotic function of RASSF1A. However RASSF1A signalling may also be offset by anti-apoptotic feedback loops. For example YAP1 activates anti-apoptotic, cell survival and cell proliferation genes by its TEAD binding.[35,36] Interestingly, binding of MST1 to RASSF1A was not directly affected by substitutions at Serine 203. The exact pathway involved in the phosphorylation-mediated apoptosis by RASSF1A is currently under investigation.

Recently it has been reported that ATM kinase regulates a RASSF1A-dependent DNA damage response, and phosphorylation of Serine 131 of RASSF1A by ATM kinase upon irradiation of cells was observed.[9] However, we found no significant effect on the phosphorylation rate when we either deleted or mutated Serine 131 (Figs. 2 and 3). This could be due to a limited activity of ATM in the IGR1 cell extract.

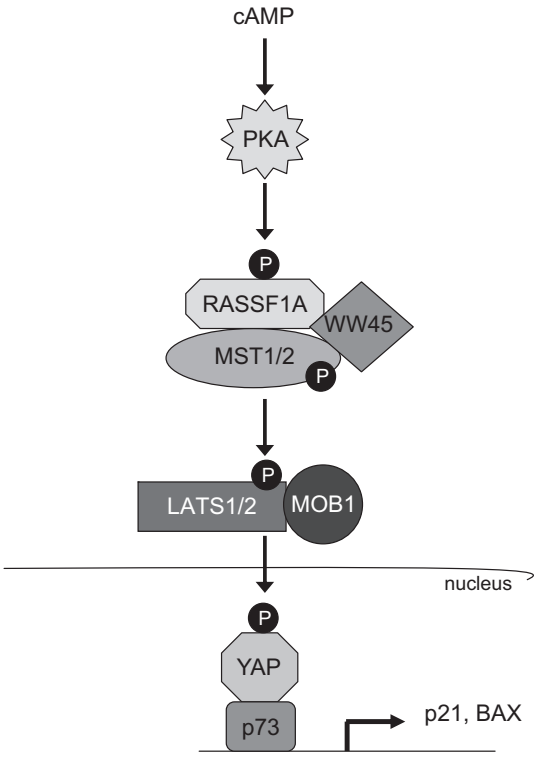

Fig. 8 – Model for PKA-mediated activation of the apoptotic RASSF-signalling pathway. Protein kinase A (PKA) activates RASSF1A and as a complex with WW45 RASSF1A regulates the proapoptotic kinases MST1/2. Subsequently MST2, activates LATS1/2, and YAP1 becomes phosphorylated by LATSs in association with MOB1. YAP1 translocates to the nucleus and forms a complex with p73 leading to the transcription of *p21* and *BAX*.

In summary we report that PKA effectively phosphorylated RASSF1A at Serine 203. Substitutions of this residue affected the microtubule stability. Moreover, RASSF1A induced apoptosis through upregulation of *p21* and *BAX*. Interestingly, the inhibition of PKA and the S203A substitution of RASSF1A resulted in a significantly reduced rate of apoptotic cells and *p21* and *BAX* induction. These findings suggest that PKA-mediated phosphorylation of RASSF1A contributes to its proapoptotic signalling.

Conflict of interest statement

None declared.

Acknowledgement

This work was supported by a Grant DA552 from the German Research Council (DFG) to Reinhard H. Dammann. This organisation had no involvement in the study design, in the acquisition, analysis and interpretation of data, in the writing of

the manuscript and in the decision to submit the manuscript for publication.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.06.128.

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