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Role of the tumor suppressor RASSF2 in regulation of MST1 kinase activity

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ABSTRACT

The tumor suppressor, RASSF2 (Ras association domain family 2), is frequently downregulated in a number of cancers. Although exogenously expressed RASSF2 induces apoptotic cell death, the precise roles of RASSF2 under pro-apoptotic conditions remain largely unknown. Here, we demonstrate that MST1 (mammalian sterile 20-like kinase 1) regulates RASSF2 protein stability. Knockdown of MST1 in cancer cells markedly destabilizes RASSF2, and Mst1-deficient mice show reduced Rassf2 protein levels in several organs. Conversely, RASSF2 activates MST1 kinase activity through formation of a RASSF2–MST1 complex, which inhibits the MST–FOXO3 signaling pathway. RASSF2 also engages the JNK pathway and induces apoptosis in an MST1-independent manner. Collectively, these findings indicate that MST1 is a major determinant of RASSF2 protein stability, and suggest that RASSF2 acts in a complex manner that extends beyond simple protein–protein association to play an important role in MST1 regulation.

Introduction

The RASSF (Ras association domain family) protein family consists of 10 members (RASSF1–10) with various isoforms [1,2], all of which share a conserved RalGDS/AF6 Ras association (RA) domain in the C-terminal (RASSF1–6) or N-terminal (RASSF7–10) region. RASSF1–6 also contain a characteristic SARAH (*Sa*lvador-*RA*SSF-*H*ippo) domain adjacent to the RA domain, and function as tumor suppressors. The RASSF2 gene is frequently downregulated in a number of different tumor types, including colorectal, gastric, breast, and lung tumors [3–7], and hepatocellular, nasopharyngeal, and oral squamous cell carcinomas [8–10]. RASSF2 has a putative nuclear localization signal (NLS) and a nuclear export signal (NES) at the N- and C-terminal regions, respectively [6,11,12]. Shuttling between the nucleus and cytoplasm, RASSF2 induces cell cycle arrest and apoptosis [9,12,13].

The pro-apoptotic protein kinases, MST1 and MST2 (*M*ammalian *St*erile 20-like kinase 1 and 2), which are key components of the Hippo signaling pathway, are involved in cell proliferation and survival [14,15]. In response to apoptotic stimuli, MST is cleaved by caspase-3, generating the N-terminal catalytic kinase fragment. This proteolytically generated MST1 kinase fragment translocates to the nucleus, where it phosphorylates histone H2B at serine 14 to subsequently cause chromatin condensation, DNA fragmentation, and apoptotic cell death [16]. The FOXO transcription factors, which regulate gene expression involved in the cell cycle, DNA-damage repair, oxidative stress, and apoptosis, have been shown to be substrates for MST kinase [17,18]. Phosphorylation of FOXO3 on serine 207 by MST1 activates FOXO3-dependent transcription and cell death [19]. This process is opposed by AKT, which phosphorylates MST1 and inhibits its activity [20].

Although RASSF1 and RASSF5 (NORE1) have been shown to interact with MST1/2 protein kinases and to play a role in cell-cycle regulation and apoptosis [21–25], little is known about the relevance of RASSF2 in MST1/2-dependent processes. However, recent studies have revealed that RASSF2 is a major binding partner of MST1/2 kinases, and can regulate MST1/2 protein stability in cancer cell lines [26]. Here, we found that RASSF2 protein stability is significantly decreased in the absence of MST1 *in vitro* and *in vivo*. In addition, we found that RASSF2 increases MST1 autophosphorylation and enhances its kinase activity toward histone H2B *in vitro*, but inhibits MST1 activity toward FOXO3 *in vivo*. Thus, RASSF2 protein stability is critically dependent on MST1, and MST1 kinase activity is regulated by RASSF2 via RASSF2-MST1 protein complex formation.

Materials and methods

Plasmid construction. cDNAs for human AKT, FOXO3, and RASSF2 (NM_014737) were cloned into pcDNA-Flag or pcDNA-HA, which were modified from the pcDNA3 vector (Invitrogen) to include an N-terminal Flag or hemagglutinin (HA) coding sequence. Expression vectors for Flag-tagged human MST1 and the kinase-dead (KD) MST1(K59R) mutant have been described previously [21].

Cell lines and reagents. 293T and U2OS cells were cultured in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (FBS). U2OS cells stably

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transfected with small interfering RNA (siRNA) against MST1 (siMST1) or control siRNA targeting GFP (siGFP) have been described previously [21]. Transfections were performed using polyethyleneimine, as standard protocol (Polysciences). Cycloheximide was obtained from Sigma–Aldrich, and wortmannin was obtained from Calbiochem.

Antibodies. Polyclonal rabbit or guinea pig anti-RASSF2 antibodies were generated against His_6-tagged recombinant full-length RASSF2 proteins, or against a KLH (keyhole-limpet-hemocyanin)-conjugated synthetic peptide (69-QMQDDNERIRPPPSSSSWH-87) specific for RASSF2. Other antibodies included anti-Flag (M2; Sigma–Aldrich), anti-HA.11 (16B12; Covance), anti-histone H2B (Upstate Biotechnology), anti-phospho-FOXO3 (S207) (Biosource), anti-β-actin (Sigma–Aldrich), and anti-GAPDH (Abcam) antibodies. Anti-MST1, anti-MST2, anti-phospho-MST (T183/T180), anti-phospho-AKT (specific for S473 or T308), and anti-FOXO3 antibodies were obtained from Cell Signaling.

Immunoprecipitation and in vitro kinase assay. 293T cells were cotransfected with wild-type or kinase-inactive mutant (KD) of Flag-MST1 in the presence or absence of HA-RASSF2. Forty-eight hours after transfection, cells were lysed with cell lysis buffer containing 50 mM Tris·Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM $MgCl_2$, 0.5% Triton X-100, protease inhibitors (1 mM phenylmethylsulfonylfluoride, 2 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin A), and phosphatase inhibitors (5 mM NaF, 5 mM β-glycerol phosphate, and 1 mM Na_3VO_4). Immunoprecipitation and *in vitro* kinase assay were performed as previously described [21]. Briefly, immunoprecipitates were washed four times with cell lysis buffer and then once with 0.5 M LiCl in PBS buffer. The washed precipitates were resuspended in kinase buffer (40 mM HEPES (pH 7.4), 20 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10 μM unlabeled ATP, 1 μCi [γ-^{32}P]ATP, and phosphatase inhibitors), and incubated with 1 μg histone H2B (Roche) for 25 min at 30 °C. Reaction mixtures were analyzed by SDS–PAGE and autoradiography.

Mst1-knockout and transgenic mice. Generation of Mst1-knockout mice was previously described [27]. $Mst1^{-/-}$; Tg mice were generated by crossing $Mst1^{-/-}$ mice with wild-type human MST1 transgenic mice.

Splenic T cell preparation. Mouse spleens were sieved through a 40-μm Cell Strainer (BD Falcon) followed by gradient centrifugation with Lymphocyte Separation Media (Mediatech Cellgro). Isolated lymphocytes were separated using CD4 or CD8a T cell isolation kits (Miltenyi Biotec) as per the manufacturer's instructions.

Results

MST1 increases RASSF2 protein stability in vitro

To identify the nature of MST1–RASSF2 interactions, we first transiently transfected 293T cells with HA-tagged RASSF2 with or without co-transfection of Flag-tagged MST1. Cells overexpressing RASSF2 with MST1 showed much higher HA-RASSF2 protein levels than did controls transfected with RASSF2 only (Fig. 1A). We further confirmed that MST1 stabilized RASSF2 protein by treating cells with the protein synthesis inhibitor, cyclohexamide (CHX). As shown in Fig. 1B, RASSF2 protein stability was profoundly increased in the presence of MST1. Consistent with a previous report [26], we also confirmed that endogenous MST1 and MST2 coimmunoprecipitated with RASSF2 proteins in U2OS cells (Fig. 1C) and HeLa cells (data not shown). Interestingly, in U2OS cells, endogenous RASSF2 appeared to bind preferentially to MST1, and this endogenous RASSF2–MST1 complex was abundant under normal conditions. Moreover, we found that cells depleted of MST1 using siMST1 exhibited significantly reduced levels of RASSF2 compared to cells transfected with siGFP controls (Fig. 1D). These results indicate that formation of a RASSF2–MST1 protein complex is likely an important factor in maintaining RASSF2 protein stability.

Mst1-mediated Rassf2 stability in vivo exhibits tissue specificity

We next investigated whether MST1 increased RASSF2 protein stability *in vivo*. For this purpose, we immunoblotted several organs from Mst1-knockout ($Mst1^{-/-}$) mice for Rassf2 [27]. Consistent with the results from MST1-depleted cells, Rassf2 levels in $Mst1^{-/-}$ mice were decreased to varying degrees in different organs (Fig. 2A). We further examined Rassf2 levels in specific cell types of $Mst1^{-/-}$ mice. Because previous reports have shown that MST1 is critical for immune system maintenance through regulation of naïve T cell homeostasis and lymphocyte trafficking *in vivo* [28,29], we focused on Rassf2 expression in lymphocytes. Consistent with the reduction of Rassf2 in total extracts of spleens of $Mst1^{-/-}$ mice (Fig. 2A), Rassf2

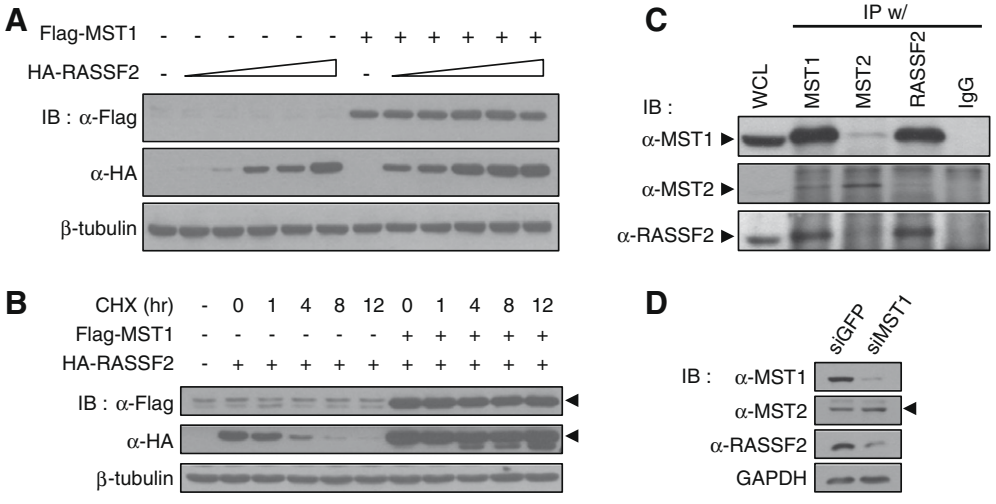

Fig. 1. MST1 regulates RASSF2 protein stability *in vitro*. (A) 293T cells were transiently cotransfected with Flag-MST1 (0.2 μg) and increasing amounts of HA-RASSF2 (0.05, 0.1, 0.5, 1, and 5 μg) as indicated. Expression of Flag-MST1 was not influenced by increasing levels of HA-RASSF2. (B) 293T cells were cotransfected with HA-RASSF2 (1 μg) and Flag-MST1 (0.2 μg) or control vector, and then 36 hours after transfection were treated with cycloheximide (CHX, 50 μg/mL) for the indicated times. (C) Lysates of U2OS cells were immunoprecipitated with antibodies to MST1, MST2, and RASSF2 (G.P), and were immunoblotted with the indicated antibodies. Pre-immune guinea pig serum (IgG) was used as an immunoprecipitation control for the guinea pig anti-RASSF2 antibody. (D) Lysates of U2OS cells stably transfected with siMST1 or siGFP (control) were immunoblotted with the indicated antibodies.

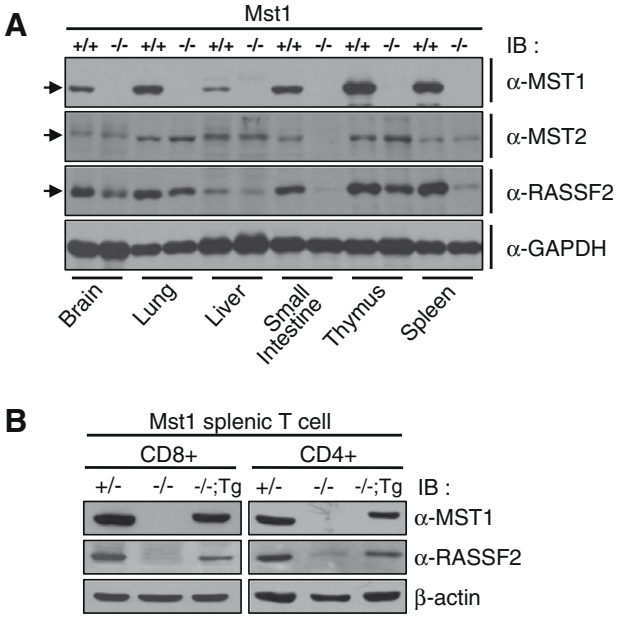

Fig. 2. Tissue-specific regulation of RASSF2 in Mst1$^{-/-}$ mice. (A) Tissue lysates from Mst1$^{+/+}$ and Mst1$^{-/-}$ mice were immunoblotted with anti-MST1, anti-MST2, or anti-RASSF2 antibodies. An anti-GAPDH antibody was used as a loading control. (B) Splenic CD8^{+} and CD4^{+} T cells from Mst1$^{+/-}$, Mst1$^{-/-}$, and Mst1$^{-/-}$; Tg mice were immunoblotted with anti-MST1 or anti-RASSF2 antibodies.

protein levels were greatly decreased in Mst1-deficient splenic CD8^{+} and CD4^{+} T cells (Fig. 2B). Moreover, this decrease in Rassf2 levels was significantly restored in Mst1$^{-/-}$; Tg mice (Fig. 2B), confirming that Mst1 is required for Rassf2 stability. Notably, we found that Rassf2 levels were not altered in organs of Mst2$^{-/-}$ mice (data not shown). Taken together, these results suggest that Mst1, but not Mst2, plays a major role in controlling Rassf2 protein stability via formation of an Mst1–Rassf2 complex, a regulatory mechanism that may depend on cell or tissue type *in vivo*.

RASSF2 induces apoptosis in association with JNK activation

Because RASSF2 is known to induce apoptotic cell death [4,30], we next investigated the mechanism of RASSF2-induced cell death, using *in vitro* kinase assays to directly examine whether the MST1–RASSF2 complex influenced MST1 kinase activity. To this end, we transfected 293T cells with wild-type Flag-MST1 or a K59R Flag-MST1(KD) mutant together with HA-RASSF2. Cell lysates were immunoprecipitated with an anti-Flag antibody, and next assayed for kinase activity using histone H2B as a substrate [16]. Consistent with a previous report [26], we found that both wild-type MST1 and mutant MST1(KD) coimmunoprecipitated with RASSF2, but only wild-type MST1 phosphorylated RASSF2 (Fig. 3A). Measurement of the autophosphorylation activity of immunoprecipitated MST1 revealed that the RASSF2–MST1 complex markedly enhanced MST1 activity (Fig. 3A and B), even though the levels of immunoprecipitated MST1 were comparable in the presence and absence of cotransfected RASSF2. In control experiments, autophosphorylation of MST1 was not detectable in immunoprecipitates prepared from cells expressing the MST1(KD) mutant. We also observed that MST1 activity toward histone H2B was greatly augmented in the presence of RASSF2 (Fig. 3A). Interestingly, overexpression of RASSF2 induced MST1 kinase activation in transfected cells in the absence of MST1-activating stimuli (e.g., Fas, staurosporine, or okadaic acid) [21]. This result was further confirmed in experiments using increasing amounts of transfected RASSF2, which showed that autophosphorylation of MST1 and phosphorylation of histone H2B were increased by RASSF2 in a dose-dependent manner (Fig. 3B). Thus, our results suggest that RASSF2 is a substrate for MST1 kinase and also enhances MST1 activity.

In previous reports, JNK activation was shown to be a prerequisite for MST1-mediated chromatin condensation during apoptosis [31]. Because we found that RASSF2 increases MST1 kinase activity and previous studies have shown that overexpressed RASSF2 induces cell death [9,30], we next examined the involvement of the JNK pathway and MST1 in RASSF2-induced apoptosis. To address this aspect, we transfected HA-RASSF2 into parental U2OS cells or U2OS cells depleted of MST1 with siMST1. As expected, overexpressed RASSF2 activated JNK pathways in association with induction of PARP and caspase-3 cleavage, indicating that RASSF2 induces cell death (Fig. 3C). Interestingly, the degree of RASSF2-mediated JNK activation, and the extent of PARP and caspase-3 cleavage, did not differ between control and MST1-depleted cells, indicating that RASSF2-mediated cell death is largely unaffected by differences in MST1 levels (Fig. 3C). One possible explanation for this is that MST2 could fully compensate for reduced MST1 activity, as MST1-depletion did not cause a reduction in MST2 levels (Fig. 1D). Alternatively, RASSF2-induced apoptosis may be inde-

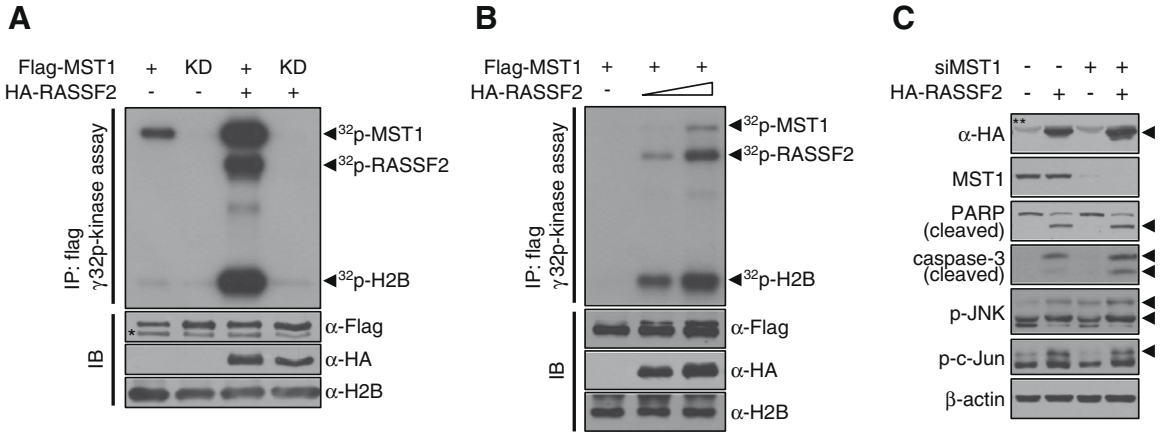

Fig. 3. RASSF2 increases MST1 activity and activates the JNK pathway. (A) 293T cells were cotransfected with wild-type or kinase-inactive (K59R) Flag-MST1 (0.2 μg), and HA-RASSF2 (1 μg). Cell lysates were immunoprecipitated with anti-Flag antibody, and the resulting immunoprecipitates were used in *in vitro* kinase assays with histone H2B as a substrate. Reactions were analyzed by SDS–PAGE. Each blot was stripped and reprobed with anti-Flag, anti-HA, and anti-histone H2B antibody (loading controls). (*denotes mouse IgG heavy chain.) (B) 293T cells were cotransfected with Flag-MST1 (0.5 μg) and increasing amounts of HA-RASSF2 (0.1 and 0.5 μg). Lysates were subjected to kinase assays as described in (A). (C) U2OS cells stably transfected with siMST1 or siGFP were transfected with HA-RASSF2 or control vector. Cell lysates were analyzed by immunoblotting with indicated antibodies (**non-specific band).

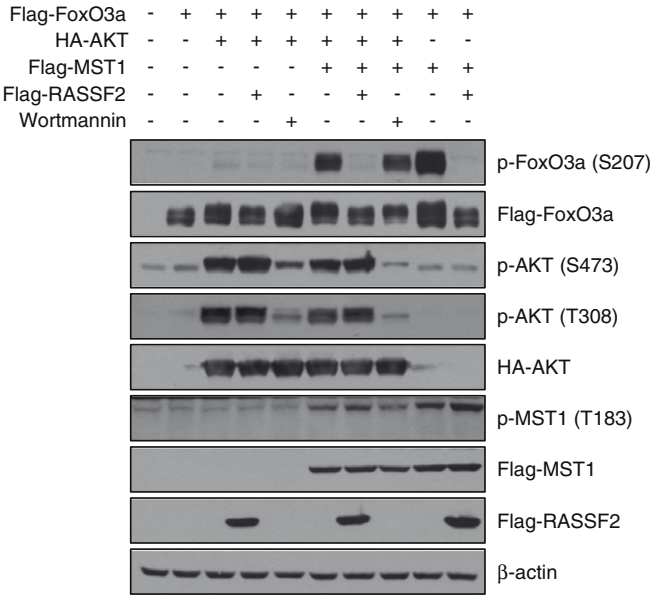

Fig. 4. RASSF2 inhibits MST1-mediated FOXO3 activation. 293T cells were cotransfected with Flag-FOXO3 (0.2 μg), HA-AKT (2 μg), Flag-MST1 (1 μg) and/or Flag-RASSF2 (1 μg) in different combinations, followed by treatment with the phosphatidylinositol 3-kinase inhibitor, wortmannin (100 nM), or equivalent amount of DMSO (vehicle control) for 30 min. Cell lysates were analyzed for FOXO3 phosphorylation. FOXO3 protein stability was increased in the presence of MST1; increased FOXO3 expression in MST1-cotransfected cells was reduced by coexpression of RASSF2 or AKT (second panel).

pendent of MST1/2, in which case, JNK activation might be a consequence rather than a cause of RASSF2-mediated apoptosis, and PARP and caspase-3 cleavage may reflect the action of RASSF2 through a separate pro-apoptotic pathway.

RASSF2 regulates the MST1–FOXO signaling pathway

Phosphorylation of FOXO proteins by MST1 promotes neuronal cell death [19,32]. FOXO proteins are also phosphorylated and inactivated by AKT [33,34]. Interestingly, AKT also phosphorylates MST1 and inhibits MST1-mediated FOXO3 nuclear translocation, promoting cell survival [20]. On the basis of these previous observations, we further investigated the regulation of the MST1–FOXO pathway by RASSF2, specifically examining the involvement of RASSF2 in MST-mediated FOXO3 phosphorylation [19,32]. To accomplish this, we cotransfected 293T cells with FOXO3, AKT, MST1, and/or RASSF2 in different combinations. We also treated cells with wortmannin (which inhibits AKT by blocking the activity of the upstream phosphatidylinositol 3-kinase) to examine the involvement of AKT activity in MST1-mediated FOXO3 phosphorylation. As shown in Fig. 4, overexpressed MST1 significantly enhanced phosphorylation of FOXO3 on serine 207. Interestingly, wortmannin treatment reduced AKT activation (Fig. 4, third and fourth panels), but did not affect the level of FOXO3 phosphorylation on serine 207 by MST1. Surprisingly, RASSF2 completely blocked MST1-mediated phosphorylation of FOXO3 on serine 207 (Fig. 4, first panel, lanes 7 and 10) without affecting AKT activity (Fig. 4, third and fourth panels, lanes 6 and 7). These results suggest that RASSF2 regulates MST1-mediated FOXO3 activation, and imply that RASSF2 may be a more essential component of the MST–FOXO signal pathway than is AKT.

Discussion

Recently, we reported that MST1 and MST2 protein kinases play key roles in early development, primitive hematopoiesis, and cell survival *in vivo* [27]. However, compared to what is known about the important functions of MST1/2 in cell proliferation and apoptosis, very little has appeared on regulators of kinase activities. Cooper and colleagues recently showed that RASSF2 stabilizes MST2 and protects it from degradation *in vivo* [26]. Consistent with this, we confirmed using mass spectrometry that MST1/2–RASSF2 binding was prominent in NIH3T3 cells (unpublished data). In the current study, we provide the first evidence that RASSF2 protein stability is also significantly dependent on MST1 protein *in vitro* and *in vivo*. Thus, it is likely that the MST–RASSF2 complex acts as a functional unit in which RASSF2 and MST1/2 reciprocally regulate each other's stability. Previous studies showing that RASSF2 regulates a number of genes involved in immune responses, hematological development, and cell–cell interactions [9,13] in which MST plays a physiological role [27–29] support this interpretation. Of interest, it has been reported that RASSF5 (NORE1) levels are significantly reduced in $Mst1^{-/-}$ T cells [28], supporting the possibility that MST1 functions as a scaffolding protein to stabilize various RASSF family proteins (RASSF1–6).

Furthermore, we showed that RASSF2 activates MST1 kinase activity, but inhibits MST1-mediated FOXO3 phosphorylation. The effect of RASSF2 on MST1-mediated histone H2B phosphorylation *in vitro* does not seem to be consistent with its effects on MST1-mediated FOXO3 phosphorylation *in vivo*. However, this apparent discrepancy is consistent with previous findings that RASSF1A is able to either activate or inhibit MST1 activity, depending on the experimental setting [21]. In addition, this result implies that RASSF2 may regulate MST1 stability in intact cells through a mechanism that is more complex than a simple association between the two proteins. It is thus possible that by recruiting other effectors into the MST1–RASSF2 complex, RASSF2 could have a dual role as an inhibitor and an activator, depending on the specific substrates of MST1. Therefore, it will be important to identify proteins that affect RASSF2 regulation of MST1.

What is the physiological significance of JNK activation during the course of RASSF2-induced apoptosis? One possibility raised by our results is that JNK activation is not caused solely by activated MST1 complexed with RASSF2. Previous reports have shown that the Rho family of GTPases plays critical roles in cytoskeletal integrity, cell proliferation, and cell-cycle regulation [35]. In colorectal cancer cell lines, RASSF2 acts via suppression of RhoA to induce disruption of the actin cytoskeleton, causing changes in cell shape and promoting apoptosis [4]. In this context, it is notable that MST kinase, which activates JNK/SAPK pathways and promotes apoptosis [36,37], can be activated by impaired actin cytoskeleton integrity [38]. As we show here for the first time, RASSF2-induced apoptosis evokes JNK pathway activation, apparently independent of MST1; thus, RASSF2 might induce JNK activation by disrupting actin structure, possibly through inhibition of RhoA. The central element in this scenario is cytoskeletal integrity, which could activate MST kinase (or other unknown kinases), leading to MST-dependent (or independent) JNK activation.

In conclusion, we provide the first evidence that MST1 is a critical regulator of RASSF2 protein stability *in vitro* and *in vivo*, and further propose that the endogenous RASSF2–MST protein complex may be a functional unit that exerts a mutually stabilizing influence on its respective partners. In addition, our data demonstrate that the tumor suppressor protein, RASSF2, functions as a novel regulator of MST1 kinase, and controls MST1 activity under pro-apoptotic conditions.

Acknowledgments

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