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Report

RAN GTPase Is a RASSF1A Effector Involved in Controlling Microtubule Organization

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Summary

RASSF1A **is a tumor suppressor gene that is inactivated by hypermethylation of its promoter region in most types of human cancers [1–3]. The incidence of spontaneous or induced tumors is significantly higher in *Rassf1a*$^{-/-}$ mice than in wild-type mice, confirming the tumor suppressor function of RASSF1A [4, 5]. RASSF1A promotes apoptosis mainly through its interaction with the proapoptotic serine/threonine STE20-like kinases MST1 and 2 [6, 7]. However, *Rassf1a*$^{-/-}$ mice do not show overt signs of deregulated apoptosis [4, 5], suggesting that other RASSF1A effectors are also critical for tumor suppression. In a proteomics screen, we identified RAN GTPase, MST1 and 2 kinases, and α- and γ-tubulin as RASSF1A-interacting proteins. We show that RASSF1A-induced microtubule hyperstability, a hallmark of RASSF1A expression [8, 9], is RAN-GTP dependent. RASSF1A promotes the accumulation of the GTP-bound form of RAN via the MST2-induced phosphorylation of RCC1. Depletion of RASSF1A results in mislocalization of RCC1 to the mitotic spindle and spindle poles, leading to mitotic spindle abnormalities and prometaphase block. A similar mitotic delay is also observed with MST2 depletion. These findings reveal a mechanism for how RASSF1A controls microtubule stability and for how its loss compromises the integrity of the mitotic spindle, leading to aneuploidy and tumorigenesis.**

Results and Discussion

RAN GTPase Is a Novel RASSF1A-Interacting Protein

In order to identify additional factors that mediate RASSF1A-induced microtubule hyperstability, we immunoprecipitated FLAG-RASSF1A transiently expressed in HEK293 cells and identified interacting proteins via 2D liquid chromatography-tandem mass spectrometry [10]. RAN GTPase (NP_006316) was identified as a novel RASSF1A-interacting protein in this screen. RAN is a small GTPase that is crucial in the regulation of nucleocytoplasmic transport [11] as well as assembly of the mitotic spindle [12]. RAN GTPase was specifically pulled down by GST-RASSF1A, but not by GST alone (Figure 1A). Endogenous RASSF1A and RAN coprecipitated in HCT116 colorectal cancer cells (Figure 1B), confirming that the RASSF1A-RAN interaction occurs at physiological levels. This interaction appears to be direct, because bacterially expressed and purified RASSF1A (GST-RASSF1A) and RAN interacted with each other in vitro (Figure 1C). When lysates from HEK293 cells transiently expressing GST-RASSF1A were loaded with the nonhydrolyzable GTP analog GTPγS or GDP prior to GST pull-down, RASSF1A interacted preferentially with RAN-GTP (Figure 1D). In order to map the RASSF1A-RAN interaction sites, GST-RAN-Q69L was coexpressed with full-length HA-RASSF1A and different truncated mutants. Only full-length HA-RASSF1A, HA-RASSF1A 1–285 aa, and to a lesser extent HA-RASSF1A 120–340 aa coprecipitated with GST-RAN (Figure 1E), suggesting that RASSF1A residues 120–285 aa mediate the interaction with RAN. The weak interaction of RASSF1A 120–340 aa with RAN further suggests that the C1 domain, located between aa 52 and 101, participates in but is not essential for RASSF1A-RAN interactions. A role for the C1 domain became more evident when we analyzed a mutation in the C1 domain of RASSF1A (C65R) that abrogates its ability to hyperstabilize microtubules [8]. This mutation also inhibited the interaction with RAN, whereas other RASSF1A mutations did not compromise RAN binding (see Figure S1 available online). This observation suggests that both the C1 domain and the RA domain are necessary for efficient RAN binding and that this interaction in turn is important for RASSF1A-microtubule association. In order to examine the cellular localization of the RASSF1A-RAN interaction, immunofluorescence staining of RASSF1A and RAN in mitotic HeLa cells was performed. Because endogenous RASSF1A is difficult to detect, we rigorously ascertained the specificity of the RASSF1A antibody (Figure S2). As expected, the majority of the RAN signal was associated with chromatin. However, as reported previously [13], a proportion of RAN also existed at the spindle poles, where it colocalized with endogenous RASSF1A (Figure 1F).

RASSF1A-Induced Microtubule Hyperstability Is RAN-GTP Dependent

Next, we examined whether the RASSF1A-induced microtubule hyperstabilization is RAN-GTP dependent. For this, we utilized the tsBN2 cell line, which harbors a temperature-sensitive mutation in the RAN guanine nucleotide exchange factor RCC1 [14]. This mutation causes the degradation of RCC1 when cells are grown at restrictive temperature (39.5°C), effectively reducing RAN-GTP levels (Figure S3A). Nocodazole treatment at the permissive temperature (Figure 1G, left panel) did not cause any noticeable difference in typical RASSF1A-induced microtubule hyperstabilization [8]. At the restrictive temperature, however, RASSF1A-induced microtubule hyperstabilization was lost and the GFP-RASSF1A signal on the microtubules

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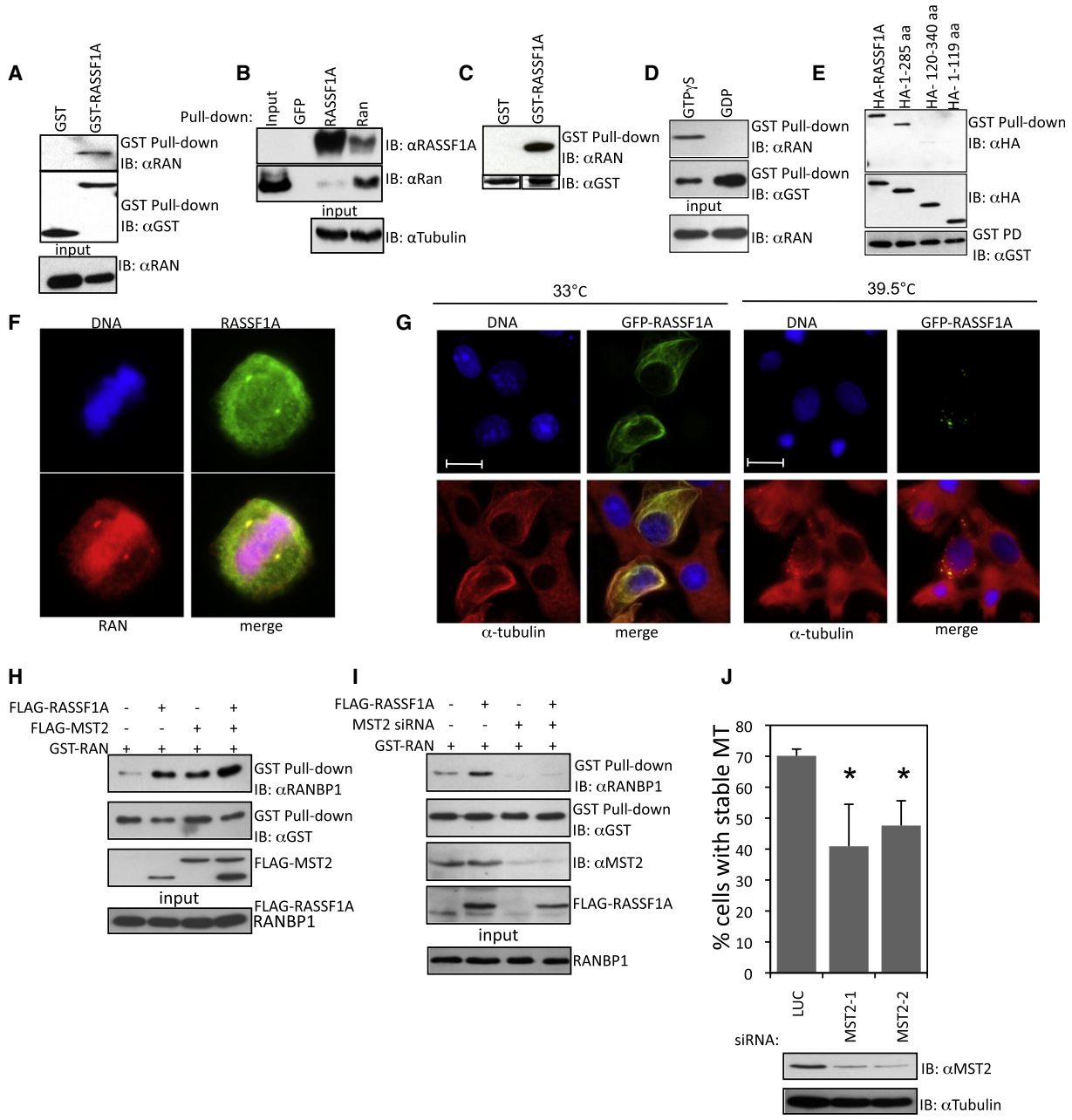

Figure 1. RASSF1A Interacts with RAN-GTP

(A) GST-RASSF1A interacts with endogenous RAN. GST or GST-RASSF1A proteins transiently expressed in HEK293 cells were pulled down and immunoblotted (IB) for associated RAN GTPase.

(B) RASSF1A and RAN GTPase interact in vivo. Endogenous RASSF1A and RAN GTPase were coimmunoprecipitated from HCT116 lysates with the respective antibodies. The input lane does not show a band for RASSF1A because the antibody used in the immunoprecipitation (eb114) does not efficiently detect RASSF1A levels in lysates. Our in-house antibody was not suitable for use in this blot because of cross-reactivity with the heavy and light chains of the antibodies used in the immunoprecipitation.

(C) Bacterially purified RAN GTPase and GST-RASSF1A interact directly in vitro.

(D) RASSF1A has higher affinity for RAN-GTP. Equal volumes of lysates generated from HEK293 cells transiently expressing GST-RASSF1A were either treated with 1 mM GDP or 100 μM GTPγS for 30 min at 30°C prior to GST pull-down and western blotting.

(E) Mapping the sites of interaction between RASSF1A and RAN GTPase.

(F) Colocalization of endogenous RASSF1A (green) and RAN GTPase (red) in methanol-fixed mitotic HeLa cells (DNA shown in blue).

(G) RASSF1A-mediated microtubule hyperstability is RAN dependent. tsBN2 cells were treated as detailed in Supplemental Experimental Procedures; only nocodazole-treated cells are shown. Scale bars represent 10 μm.

(H) Expression of FLAG-RASSF1A and FLAG-MST2 enhances the ability of GST-RAN to associate with the RAN-GTP-specific binding protein RANBP1.

(I) siRNA-mediated knockdown of MST2 interferes with GST-RAN's ability to interact with RANBP1.

(J) Top: RASSF1A ability to hyperstabilize the microtubules (MT) is MST2 dependent. Error bars represent the standard deviation of three experiments. *p = 0.015 and 0.005 by t test for Luc siRNA versus MST2-1 and MST2-2 siRNAs, respectively. Bottom: the extent of MST2 knockdown was verified by western blotting.

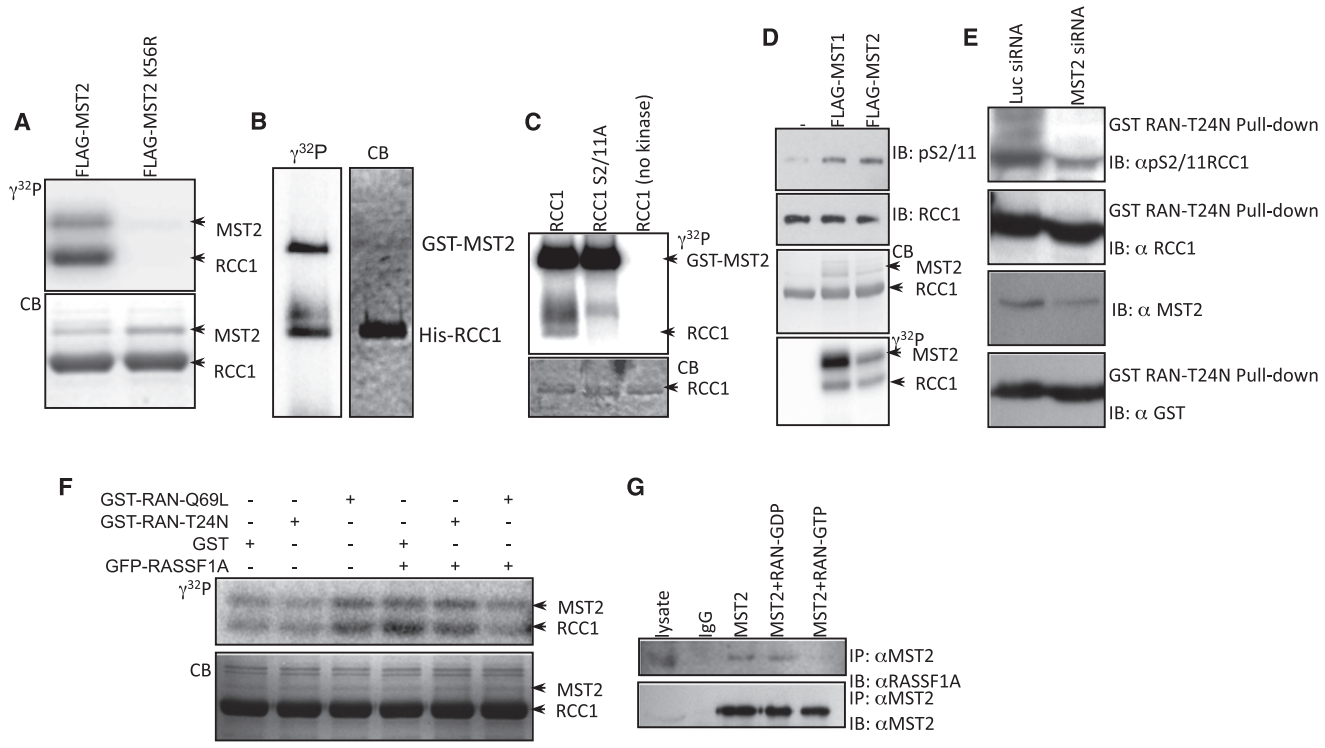

Figure 2. MST2 Phosphorylates RCC1 In Vitro and In Vivo

(A) HEK293 cells were transfected with FLAG-MST2 or the kinase-inactive mutant FLAG-MST2-K56R. MST2 and MST2-K56R were immunoprecipitated with FLAG-agarose beads and incubated with recombinant RCC1 protein as substrate in the presence of [γ-^{32}P]ATP. After separation on SDS-PAGE, the gel was Coomassie stained, and phosphorylation was detected by phosphoimaging. CB indicates Coomassie-stained gels.

(B) Purified GST-MST2 (Cell Signaling) can also directly phosphorylate recombinant RCC1.

(C) FLAG-RCC1 and FLAG-RCC1 S2/11A were purified from phosphatase-treated HEK293 lysates (lambda phosphatase, Cell Signaling; 100 U/50 μl lysate at 30°C for 20 min) via FLAG-agarose and used in an in vitro kinase assay with 27 mU recombinant MST2 as a kinase.

(D) Immunoprecipitated FLAG-MST1 and MST2 were used to phosphorylate purified RCC1.

(E) Knockdown of MST2 by siRNA oligonucleotides results in reduced phosphorylation at RCC1 S2/11A. Cells were transfected with siRNA oligonucleotides against MST2 or Luc (control). Endogenous RCC1 proteins were pulled down with GST-RAN-T24N and western blotted with the indicated antibodies.

(F) The effect of modulating RAN GTP/GDP ratios on MST2 activity. GST, GST-RAN-T24N, and/or GST-RAN-Q69L were expressed in the indicated combinations in HEK293 cells. Forty-eight hours later, the cells were lysed, and immunoprecipitated endogenous MST2 was assayed for its ability to phosphorylate recombinant His-RCC1 in vitro.

(G) The endogenous MST2-RASSF1A interaction is disrupted by RAN-GTP. One milligram of each HeLa cell lysate was immunoprecipitated with MST2 antibody in the presence of 2 μM preloaded RAN-GTPγS or RAN-GDP before immunoblotting with the indicated antibodies.

became punctate or diffuse (Figure 1G, right panel; only nocodazole-treated cells are shown). Furthermore, GTPγS loading of lysates selectively increased the interaction between GFP-importin β and GST-RAN whereas GDP loading had no effect, showing that importin β can be used as a probe to detect RAN-GTP (Figure S3B). Transfection of FLAG-RASSF1A increased the association between GST-RAN and GFP-importin β expressed in HEK293 cells (Figure S3C), indicating that RASSF1A increases RAN-GTP levels. Furthermore, GST-importin β also associated with higher levels of endogenous RAN, i.e., RAN-GTP, in the presence of HA-RASSF1A (Figure S3D). Taken together, these results suggested that RASSF1A can induce RAN-GTP loading and that the ability of RASSF1A to stabilize microtubules depends critically on RAN-GTP.

MST2 Mediates the Effects of RASSF1A on the RAN Pathway

In our proteomic screen, we found RASSF1A associating with both RAN and MST1/2. To elucidate the role of MST2, we tested the effect of MST2 expression on RAN-GTP levels in HEK293 cells. RANBP1 is a RAN-binding protein that binds specifically to RAN-GTP [15, 16] and hence can be used to monitor RAN-GTP levels. Expression of FLAG-MST2 enhanced RAN-GTP levels, and coexpression of FLAG-RASSF1A and FLAG-MST2 cooperated to further increase the levels of RAN-GTP (Figure 1H). Conversely, siRNA-mediated knockdown of MST2 strongly reduced both the basal and RASSF1A-induced levels of RAN-GTP (Figure 1I). These results suggested that MST2 cooperates with RASSF1A to enhance RAN-GTP levels. Because high RAN-GTP levels are responsible for RASSF1A-induced microtubule hyperstability, depletion of MST2 should interfere with the ability of RASSF1A to enhance RAN-GTP levels and consequently reduce its ability to protect microtubules against nocodazole-induced depolymerization. Cells with FLAG-RASSF1A expression exhibited the typical circular bundles of stable microtubules (Figure S4). Depletion of MST2 (Figure 1J; Figures S4 and S5) caused a significant reduction in the number of cells with stable microtubules (Figure 1J; $p = 0.015$ and 0.005 by t test for Luc siRNA versus MST2-1 and MST2-2 siRNA, respectively). This indicated that MST2 is required for RASSF1A-induced RAN-GTP loading and microtubule hyperstability, prompting further investigation of this role.

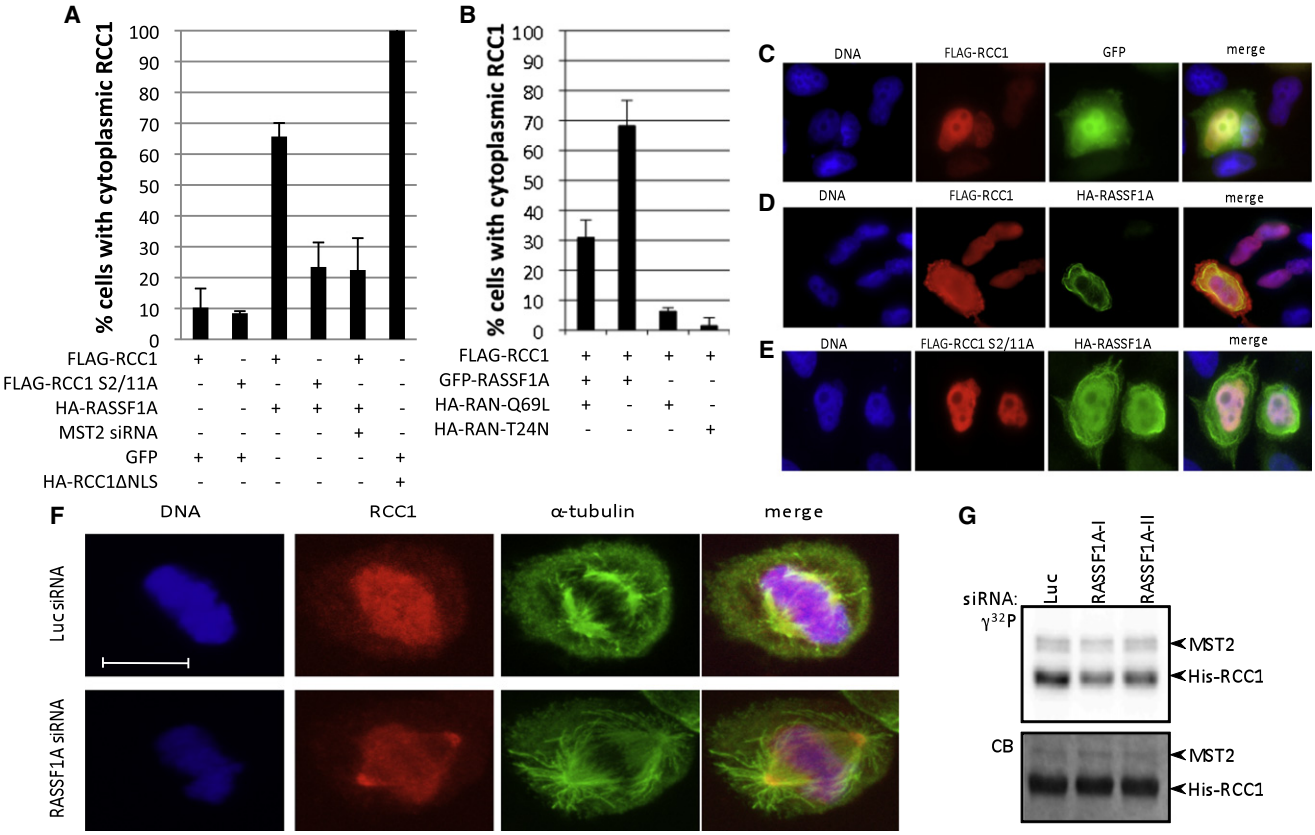

Figure 3. RASSF1A Regulates RCC1 Localization in an MST2-Dependent Manner

(A and B) RASSF1A traps RCC1 in the cytoplasm in an MST2-dependent manner (A) that is inhibited by the expression of the RAN-Q69L mutant (B). Error bars represent the standard deviation of three experiments.

(C–E) Representative immunofluorescence images used to obtain the quantifications shown in (A) and (B).

(F) Mitotic cells from Luc or RASSF1A siRNA-transfected HeLa cells were methanol fixed prior to staining with RCC1 antibody (red) and α-tubulin antibody (green). DAPI was used to stain DNA. Scale bar represents 10 μm.

(G) The effect of RASSF1A depletion on MST2's ability to phosphorylate RCC1 in mitotic cells. HeLa cells were transfected with the indicated siRNA oligonucleotides and arrested at mitosis by nocodazole. Endogenous MST2 was immunoprecipitated and used in the kinase assay as in Figure 2.

MST2 Phosphorylates RCC1

It was recently shown that in mitotic cells, RCC1 localization, chromatin association, and activity in generating RAN-GTP are controlled by phosphorylation of RCC1's N terminus, in particular serines 2 and 11, by CDK1 [17, 18]. We therefore tested whether purified recombinant human His-tagged RCC1 was a substrate for MST2. FLAG-tagged MST2 immunoprecipitated from HEK293 cells was able to autophosphorylate and also efficiently phosphorylated His-RCC1, whereas the kinase-dead mutant MST2-K56R failed to phosphorylate itself or His-RCC1 (Figure 2A). Recombinant purified MST2 also could phosphorylate purified His-RCC1, suggesting that RCC1 is a direct substrate (Figure 2B). The ability of MST2 to phosphorylate a mutant FLAG-RCC1, S2/11A, was greatly reduced (Figure 2C), demonstrating that S2/11 sites are targeted by MST2. Using an antibody generated against phospho-S2/11 in RCC1 [18], we found that these two residues were also efficiently phosphorylated by MST1 and MST2 (Figure 2D), further supporting that S2 and/or S11 are genuine MST2 phosphorylation targets. Furthermore, we utilized the in vitro purified GST-RAN-T24N mutant, which has very high affinity for RCC1, to enrich endogenous RCC1 from HEK293 cell lysates and examine its phosphorylation status with the phospho-S2/11 RCC1 antibody. RCC1 was phosphorylated on S2/11 in asynchronous HEK293 cells (Figure 2E). However, the levels of phospho-RCC1 decreased after MST2 knockdown (Figure 2E), further supporting that RCC1 is an endogenous MST2 substrate.

It is important to understand the effect of RASSF1A expression on MST2-mediated RCC1 phosphorylation as well as to examine the consequences of RAN-GTP and RAN-GDP levels on this process. Therefore, we immunoprecipitated endogenous MST2 from lysates of HEK293 cells expressing GST-RAN-T24N, GST-RAN-Q69L, or GST alone in the presence or absence of RASSF1A (Figure 2F). Interestingly, in the absence of RASSF1A, endogenous MST2 kinase activity toward recombinant His-RCC1 was significantly higher when the constitutively active RAN-Q69L mutant was expressed as compared to the dominant-negative RAN-T24N mutant. This stimulation of MST2 kinase activity by RAN-Q69L would constitute a positive feedback loop from RAN to MST2. The presence of RASSF1A resulted in increased MST2 activation and RCC1 phosphorylation, except where GST-RAN-Q69L was coexpressed. The latter result was unexpected and suggests that in the presence of RASSF1A, a negative feedback loop operates from RAN-GTP to MST2. Because RASSF1A interacts with and activates MST2 in cells [6, 7, 19], we tested the possibility that the disruption of the RASSF1A-MST2 complex by

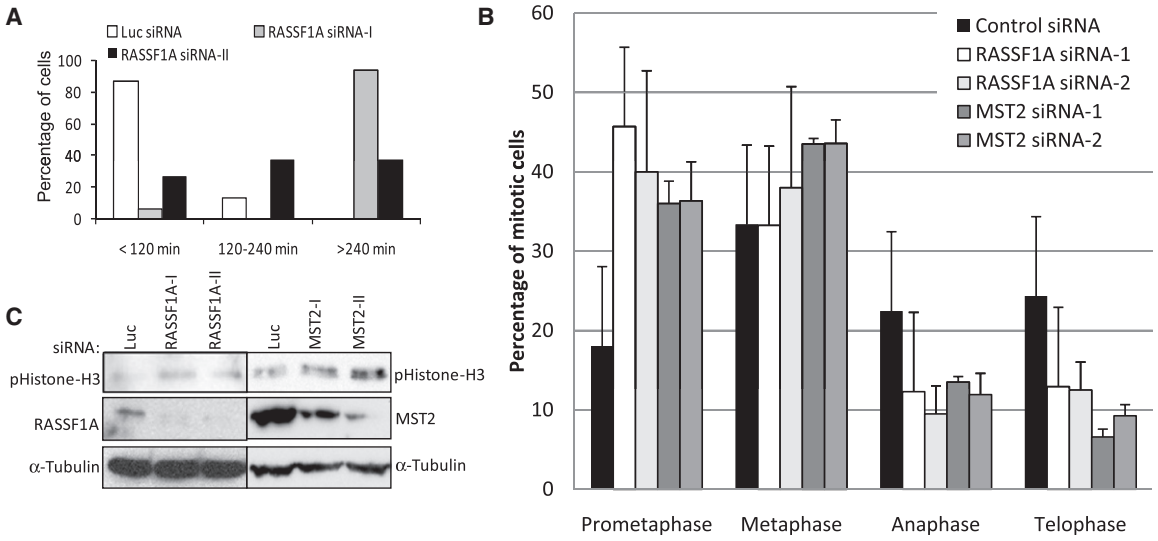

Figure 4. Knockdown of RASSF1A Causes Mitotic Delay

(A) Mitotic cells from different treatments were analyzed for the time between nuclear envelope breakdown and anaphase (n = 15). The bar chart represents the percentage of cells that divided within the time frame shown in the x axis.

(B) Following visualization of α-tubulin and DNA, the percentage of cells at the different stages of the mitotic cycle was determined by scoring ~200 cells. Error bars represent the standard deviation of three experiments. There was a statistically significant difference ($p < 0.05$ by t test) at the prometaphase stage between control and RASSF1A or MST2 siRNA-transfected cells.

(C) Mitotic delay is also evident by the elevated levels of phosphohistone H3 in RASSF1A- or MST2-depleted HeLa cells. α-tubulin was used as a loading control.

activated RAN-GTP could be part of this feedback loop. Interestingly, RAN-GTPγS, but not RAN-GDP, interfered with the endogenous RASSF1A-MST2 association (Figure 2G), suggesting that activated RAN may exert the negative feedback by disrupting the interaction between RASSF1A and MST2.

RASSF1A Regulates RCC1 Localization in Interphase and Mitotic Cells

During mitosis, RCC1 phosphorylation at S2/11 in its nuclear localization signal releases RCC1 from importin α to permit subsequent RCC1 mobility and chromatin association [17, 18]. In interphase cells, the inhibition of importin α binding by nuclear localization signal phosphorylation would be expected to compromise the nuclear transport of RCC1 and lead to accumulation in the cytosol. Because RASSF1A expression could stimulate MST2-mediated RCC1 phosphorylation on S2/11 in interphase cells (Figure 2), we investigated how RASSF1A expression affects RCC1 localization in interphase cells (Figure 3). FLAG-RCC1 was coexpressed with HA-RASSF1A in HeLa cells and examined by immunofluorescence. HA-RASSF1A expression trapped a substantial fraction of FLAG-RCC1 in the cytoplasm (Figures 3A–3E). Quantification of this effect showed that around 65% of cells expressing HA-RASSF1A and FLAG-RCC1 exhibited this phenotype, compared to only 10% in GFP- and FLAG-RCC1-transfected cells (Figures 3A, 3C, and D). The cytosolic retention of RCC1 was dependent on MST2 and S2/11 phosphorylation, because either siRNA-mediated knockdown of MST2 or transfection of RCC1-S2/11A mutant efficiently blocked the RASSF1A-induced retention of RCC1 in the cytosol (Figures 3A and 3E). Thus, in interphase cells, the phosphorylation of S2/11 by MST2 interferes with nuclear transport of RCC1. RAN-Q69L expression interfered with RASSF1A-induced cytosolic retention (Figure 3B), confirming the negative feedback loop hypothesis based on the findings that RAN-Q69L inhibited both RASSF1A-MST2 association and RASSF1A-induced MST2 kinase activity toward RCC1 (Figures 2F and 2G). Given the critical role of S2/11 phosphorylation for RCC1 function during mitosis [17, 18], we also tested the effects of RASSF1A knockdown on RCC1 localization in mitotic HeLa cells (Figure 3F). RCC1 was normally associated with the chromatin. However, in RASSF1A-depleted cells, RCC1 associated with the spindles or the spindle poles, with generally less signal associated with the chromosomes (Figure 3F, lower panel; 65% of metaphase cells counted, as opposed to 10% in control transfected cells; 20 metaphase cells counted over two independent experiments). Knockdown of RASSF1A in HeLa cells also reduced the ability of MST2 to phosphorylate RCC1 (Figure 3G).

Given such a dramatic effect on RCC1 localization, it was expected that RASSF1A depletion would be associated with mitotic abnormalities (Figure 4). Time-lapse video microscopy of HeLa cells stably expressing GFP-histone H2B [20, 21] following RASSF1A depletion showed that the majority of cells failed to proceed into anaphase even after 4 hr of microscopy (Figure 4A; Figure S6A; Movies S1 and S2). Furthermore, RASSF1A depletion was associated with a significant accumulation of cells at the prometaphase stage (Figure 4B). Importantly, this effect could be mediated through MST2, because knockdown of MST2 caused a similar extent of prometaphase delay (Figure 4B). The presence of this mitotic delay was further supported by the enhanced phosphorylation of histone H3 in RASSF1A- or MST2-depleted cells compared to controls (Figure 4C). Further mitotic abnormalities observed are detailed in Figure S6.

The RASSF1A/MST2/RAN GTPase Complex

The identification of RAN GTPase and MST2 in complex with RASSF1A suggests novel functions for MST2 and provides insight into RASSF1A mechanisms of action. In interphase cells, RASSF1A overexpression traps a fraction of RCC1 in

the cytoplasm. This is due to hyperphosphorylation of the nuclear localization signal of RCC1 mediated by cytoplasmic RASSF1A-associated MST2, which would prevent binding to importin α and shuttling into the nucleus. This could augment the normally low levels of RAN-GTP in the cytosol, permitting RASSF1A to stabilize interphase microtubules. In mitosis, the phosphorylation of RCC1 releases it from the importin complex, allowing it to stably attach to the chromosomes. Here, RCC1 generates RAN-GTP, causing the steep gradient of RAN-GTP/RAN-GDP that is required for mitotic spindle formation [22]. The knockdown of RASSF1A in mitotic cells reduces the ability of MST2 to phosphorylate RCC1, abrogating the stable association of RCC1 with chromosomes and causing redistribution to spindle poles instead (Figure S7). In normal mitosis, this is where both RASSF1A and a portion of RAN are found (Figure 1F). This gives rise to an interesting speculation (Figure S8) that during mitosis, the spindle pole may serve as a source for MST2 activation, which phosphorylates RCC1, thereby promoting stable binding of RCC1 to chromosomes. The ability of RAN-GTP to efficiently disrupt the RASSF1A-MST2 interaction (Figure 2G) could serve as a feedback mechanism that constrains the activation of RCC1 both in time and in space by diverting active RCC1 away from the spindle poles to the chromosomes. Thus, the downregulation of RASSF1A expression in many human cancers may contribute to the commonly observed chromosomal instability and subsequent tumorigenesis.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, eight figures, and two movies and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01235-4.

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