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Report

RASSF1A Is Part of a Complex Similar to the *Drosophila* Hippo/Salvador/Lats Tumor-Suppressor Network

Cai Guo,[1] Stella Tommasi,[1] Limin Liu,[1] Jiing-Kuan Yee,[2] Reinhard Dammann,[3] and Gerd P. Pfeifer[1,*]
[1] Division of Biology and
[2] Division of Virology
Beckman Research Institute of the City of Hope
Duarte, California 91010
[3] Medical Faculty
Martin-Luther-University Halle-Wittenberg
06097 Halle/Saale
Germany

Summary

The Ras Association Domain Family 1A (*RASSF1A*) gene is one of the most frequently silenced genes in human cancer. RASSF1A has been shown to interact with the proapoptotic kinase MST1. Recent work in *Drosophila* has led to the discovery of a new tumor-suppressor pathway involving the *Drosophila* MST1 and MST2 ortholog, Hippo, as well as the Lats/Warts serine/threonine kinase and a protein named Salvador (Sav). Little is known about this pathway in mammalian cells. We report that complexes consisting of RASSF1A, MST2, WW45 (the human ortholog of Sav), and LATS1 exist in human cells. MST2 enhances the RASSF1A-WW45 interaction, which requires the C-terminal SARAH domain of both proteins. Components of this complex are localized at centrosomes and spindle poles from interphase to telophase and at the midbody during cytokinesis. Both RASSF1A and WW45 activate MST2 by promoting MST2 autophosphorylation and LATS1 phosphorylation. Mitosis is delayed in Rassf1a$^{-/-}$ mouse embryo fibroblasts and frequently results in cytokinesis failure, similar to what has been observed for LATS1-deficient cells. RASSF1A, MST2, or WW45 can rescue this defect. The complex of RASSF1A, MST2, WW45, and LATS1 consists of several tumor suppressors, is conserved in mammalian cells, and appears to be involved in controlling mitotic exit.

Results and Discussion

RASSF1A is one of the most frequently silenced genes in human cancer [1, 2], and *Rassf1a*-targeted mice are prone to spontaneous and induced tumorigenesis [3]. RASSF1A interacts with the proapoptotic kinase MST1 [4]. Recent work in *Drosophila* has led to the identification of a new tumor-suppressor pathway involving the *Drosophila* MST1 and MST2 ortholog, Hippo, as well as the Lats/Warts serine/threonine kinase and a protein named Salvador (Sav) [5–10]. Interestingly, RASSF1A, MST1, MST2, and Sav all contain a conserved C-terminal domain, which has been referred to as the SARAH (Salvador, RASSF1, Hippo) domain [11]. This domain is required for the interaction of RASSF1A with MST1 [4]; it may also be the module for interaction of the other proteins of the putative complex and possibly form a trimerization motif [11]. The identification of a *Drosophila* tumor-suppressor pathway involving Hippo/MST, Salvador/WW45, and LATS kinase suggests the possibility that a similar pathway exists in mammalian cells and that this pathway includes the RASSF1A tumor suppressor. However, the existence of such a complex has not yet been demonstrated.

RASSF1A Associates with WW45 and MST2

Mammalian MST1 kinase has been reported to associate with RASSF1A [4, 12], and MST2 was shown to interact with WW45 in vitro and after coexpression [13]. We first asked whether there is an interaction between human RASSF1A and WW45. Expression constructs of the two proteins were cotransfected into COS7 cells. HA-WW45 could be coprecipitated with GST-RASSF1A (Figure 1A). A strong interaction between RASSF1A and MST2 was also observed (Figure 1B).

To investigate the effect of MST2 on the interaction between RASSF1A and WW45, we transfected different amounts of Flag-MST2 into COS7 cells together with GST-RASSF1A and HA-WW45. The interaction between GST-RASSF1A and HA-WW45 was increased by MST2 coexpression (Figure 1B). We next tried to determine whether the C-terminal SARAH domains are required and sufficient for the interaction between RASSF1A and WW45. Different HA- and GST-tagged deletion constructs of WW45 were made and cotransfected with full-length GST- or Flag-tagged RASSF1A. The data suggest that WW45's C terminus (amino acids 199–383) is necessary for binding to RASSF1A, whereas the N terminus (amino acids 1–267 and 1–320) is not. The C-terminal SARAH domain of WW45 (amino acids 321–383) was sufficient for binding to RASSF1A (see Figure S1 in the Supplemental Data available online). Next, we mapped the interaction domain(s) on RASSF1A with different RASSF1A deletion constructs (Figure S2). Surprisingly, a putative binding region appeared to be within amino acids 167–193 of RASSF1A because RASSF1A 1–193 could bind to WW45. whereas RASSF1A 1–167 could not. However, a construct with an internal deletion of this region could still bind to WW45. When the RA domain (amino acids 194–288) was present, such as in construct RASSF1A 1–288 or 120–288, the interaction of RASSF1A with WW45 was strongly reduced. Our explanation is that the RA domain may inhibit binding between RASSF1A 1–193 and WW45 unless the SARAH domain is also present. Importantly, the SARAH domain of RASSF1A alone was sufficient to bind to WW45 (Figure S2). We interpret this to mean that there are two binding sites for WW45 on RASSF1A and that the SARAH domain is the dominant one.

*Correspondence: gpfeifer@coh.org

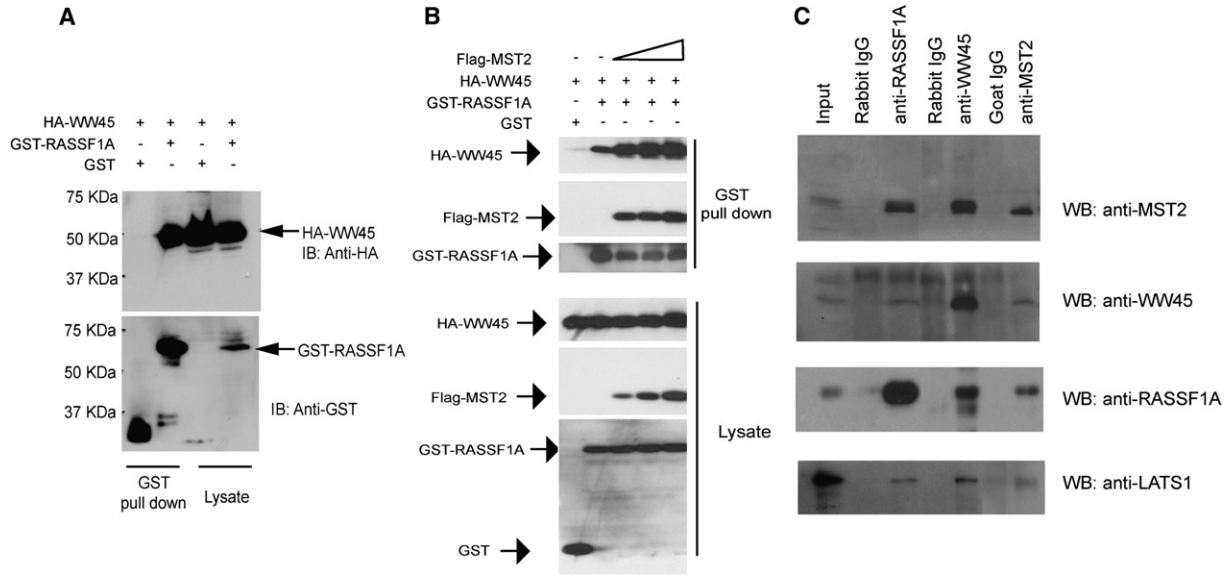

Figure 1. RASSF1A Interacts with Human WW45 and MST2

(A) Interaction of RASSF1A and WW45. A construct that expresses GST or GST-RASSF1A was cotransfected with HA-tagged WW45 into COS7 cells. After 48 hr, cells were lysed, and lysates were precipitated with a glutathione affinity matrix. The precipitates and lysates were subjected to 10% SDS-PAGE, and western blots were done with HA or GST antibodies.

(B) Effect of MST2 on the interaction between RASSF1A and WW45. GST-RASSF1A (4 μg), HA-WW45 (4 μg), and different amounts (0.4, 0.8, and 2 μg) of Flag-MST2 were transfected into COS7 cells. After 48 hr, cells were lysed, and lysates were adsorbed onto glutathione affinity gels followed by the indicated western blots.

(C) Interactions of endogenous RASSF1A, MST2, WW45, and LATS1. HeLa cell lysates were incubated with either normal control IgG or the indicated antibodies against MST2, RASSF1A, or WW45. Precipitated proteins and cell lysates were subjected to 8% SDS-PAGE followed by western blotting with MST2, WW45, RASSF1A, or LATS1 antibodies.

RASSF1A, MST2, WW45, and LATS1 Form Complexes In Vivo

In *Drosophila*, the homologs of MST1, MST2, WW45, LATS1, and LATS2 form a complex and participate in a novel tumor-suppressor pathway [5–10]. Therefore, we examined whether a similar complex exists in mammalian cells. First, we developed antibodies against RASSF1A and human WW45 (Figure S2) suitable for immunoprecipitation (Figure 1C). Different coimmunoprecipitations of the endogenous proteins were performed in HeLa cells. RASSF1A, WW45, and LATS1 all can be co-immunoprecipitated with MST2 antibody. RASSF1A, MST2, and LATS1 were associated with immunoprecipitated WW45. Moreover, when RASSF1A antibody was used for immunoprecipitation, MST2, WW45, and LATS1 were all present in the precipitated endogenous protein complexes (Figure 1C). From these results, we conclude that endogenous protein complexes including RASSF1A, WW45, MST2, and LATS1 exist in mammalian cells.

Effect of RASSF1A on MST2 Activation and LATS1 Phosphorylation

Although recombinant RASSF1A appears to inhibit MST1 and MST2 kinase activity in vitro, the effect of RASSF1A on MST1 and MST2 activity in transfected cells may involve either stimulation or inhibition [12, 14]. In order to further investigate this issue, we cotransfected RASSF1A with MST2 in COS7 cells to determine whether RASSF1A can promote the activation of MST2. A phosphospecific MST antibody that recognizes T180-phosphorylated MST2 was used [15]. Autophosphorylation of MST2 on threonine 180 was increased by RASSF1A cotransfection (Figure 2A). Because MST2 T180 is a critical residue for kinase activity [15], it was thus demonstrated that RASSF1A enhances MST2 activation. Transfection of WW45 alone (in the absence of RASSF1A) also activated MST2, but to a lesser extent than RASSF1A alone or a combination of RASSF1A and WW45 (Figure 2A). We then assessed MST2 activation in response to staurosporine treatment in wild-type and Rassf1a-knockout mouse embryo fibroblasts (MEFs) by using the P-MST antibody. Staurosporine is one of only a few agents able to activate endogenous MST1 and MST2 [15]. Although staurosporine efficiently activated MST1 and MST2, no difference in the absence of Rassf1a was observed (data not shown). Likewise, Rassf1a-deficient MEFs were not more or less sensitive than wild-type MEFs to DNA-damaging agents and microtubule-targeting drugs and did not display significant differences in apoptosis induction by these agents (S.T. and G.P.P., unpublished data).

It has been reported that MST2 phosphorylates LATS1 [13]. We considered that—if RASSF1A activates MST2—it could also promote LATS1 phosphorylation. Next, GST-RASSF1A was cotransfected with HA-LATS1 and Flag-MST2. We performed IP kinase assays to test whether RASSF1A, dependent on MST2, can enhance LATS1 phosphorylation. Transfection of active Flag-MST2 led to MST2 autophosphorylation (Figure 2B). MST2 could phosphorylate LATS1, as well as the kinase-dead form of LATS1 (Figure 2B, lanes 1, 2, 5, 6, 9, 10, 13, and 14). Kinase-dead MST2 served as a control showing that phosphorylation of LATS1 was

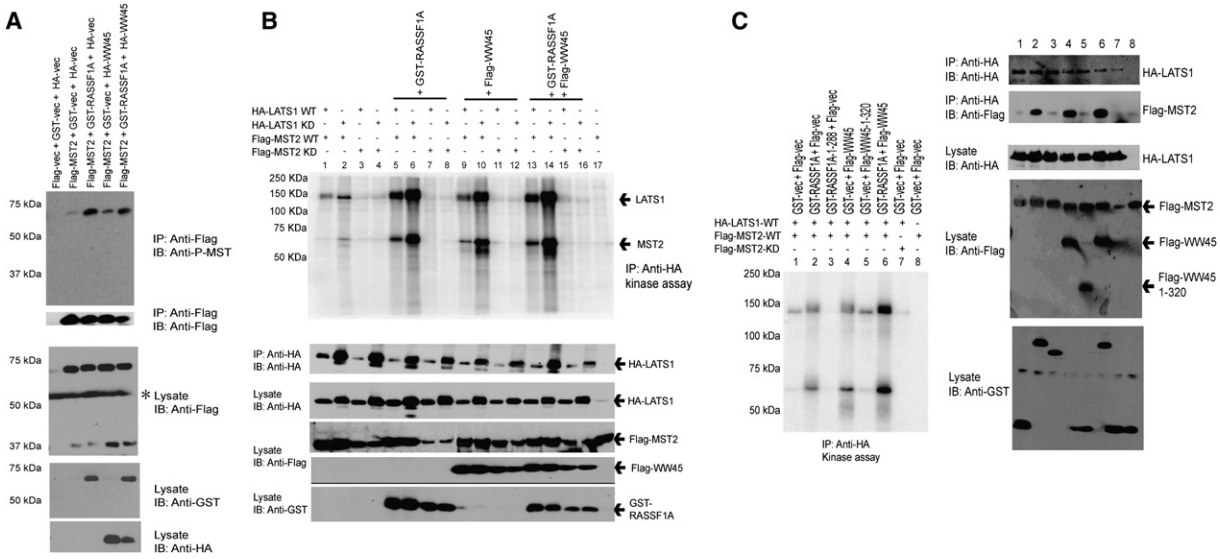

Figure 2. Effect of RASSF1A on MST2 and LATS1 Phosphorylation

(A) Effect of RASSF1A on MST2 phosphorylation. Flag-MST2 was cotransfected with plasmids expressing GST, GST-RASSF1A, HA-tag, or HA-WW45 into COS7 cells. After 48 hr, cells were lysed and precipitated with anti-Flag affinity gel. The precipitates were examined by anti-P-MST (recognizing MST2-T180P) blot. Expression of Flag-MST2, HA-WW45, and GST-RASSF1A was determined by anti-Flag, anti-HA, and anti-GST blots. The fragment near 36 kDa on the anti-Flag blots is a caspase-derived cleavage fragment of MST2. Its phosphorylated form was detectable only after longer exposure of the top gel panel. An asterisk denotes a nonspecific band.

(B) Effect of RASSF1A on MST2 activation and LATS1 phosphorylation. 293T cells were cotransfected with HA-LATS1 wild-type or HA-LATS1 kinase-dead; Flag-MST2 wild-type or Flag-MST2 kinase-dead; and GST-RASSF1A and/or Flag-WW45. Note that the kinase-dead LATS1 construct contains two consecutive HA tags, leading to an enhanced signal. Forty-eight hours after transfection, cells were lysed, and each precipitation was performed with HA antibody and protein A/G agarose beads. Precipitates were subject to an in vitro kinase assay as described in the Supplemental Experimental Procedures. Samples were separated by 8% SDS-PAGE followed by autoradiography. The indicated blots were performed so that the expression levels of the proteins could be determined.

(C) Effect of SARAH-deleted RASSF1A and WW45 constructs on MST2 activation and LATS1 phosphorylation. 293T cells were cotransfected with an HA-LATS1 wild-type construct, Flag-MST2 wild-type or Flag-MST2 kinase-dead plus GST-RASSF1A and/or Flag-WW45, full-length or SARAH-domain-deleted constructs. Forty-eight hours after transfection, cells were lysed, and each precipitation was performed with HA antibody and protein A/G agarose beads. Precipitates were subject to an in vitro kinase assay. The indicated blots were performed so that the expression levels of the proteins could be determined.

due to MST2 activity. This confirmed that the two proteins are associated with each other functionally. MST2 could be coprecipitated in a stable complex with LATS1 only in the presence of SARAH-domain-containing RASSF1A and/or WW45 (Figure 2C; lanes 2, 4, and 6); RASSF1A and WW45 combined had the strongest effect. Substantially increased LATS1 phosphorylation was observed when RASSF1A was cotransfected (Figure 2B, compare lanes 5, 6, 13, and 14 with lanes 1 and 2). WW45 alone also enhanced MST2 activity (Figure 2B, lanes 9 and 10), but RASSF1A and WW45 combined had an additional effect (Figure 2B, lanes 13 and 14; Figure 2C, lane 6). The data indicate that RASSF1A and WW45 promote LATS1 phosphorylation by recruiting MST2 into the complex. Because no specific substrate of LATS1 is known, we could not directly test whether LATS1 kinase activity is increased by MST2. We next determined the role of the SARAH domains in MST2 activation by RASSF1A and WW45. As shown in Figure 2C, RASSF1A and WW45 with a deleted SARAH domain were incapable of MST2 activation.

RASSF1A, MST2, WW45, and LATS1 Localize to the Centrosomes and Midbody

Human LATS1 localizes at centrosomes and the spindle apparatus in mitotic cells [16]. Because RASSF1A, WW45, MST2, and LATS1 were associated with each other (Figure 1), we investigated their subcellular localization patterns. All four proteins (RASSF1A, WW45, MST2, and LATS1) colocalized with γ-tubulin bright spots in interphase and mitotic cells (Figure 3), supporting the notion that the four proteins form a centrosomal complex. Anaphase localization of the four proteins is shown in Figure S3. Colocalization experiments were possible between MST2 (goat antibody) and the other three proteins (rabbit antibodies) and are shown in Figure S4. Interestingly, these proteins were localized at the midbody or the contractile ring during cytokinesis, which indicates that the complex may be important for the completion of mitosis. Whereas MST2 and RASSF1A were consistently observed at the midbody in HeLa cells, WW45 and LATS1 were localized at the contractile ring in most cells in late mitosis. However, we also observed anti-RASSF1A staining at both the contractile ring and midbody in human fibroblasts (data not shown).

Mitotic Defects in Rassf1a-Knockout Cells

LATS1 has been described as a kinase involved in the mitotic-exit network [17]. Given its cellular localization (Figure 3) and its effect on LATS1 phosphorylation (Figure 2), we considered the possibility that RASSF1A may also be involved in mitotic progression. Using

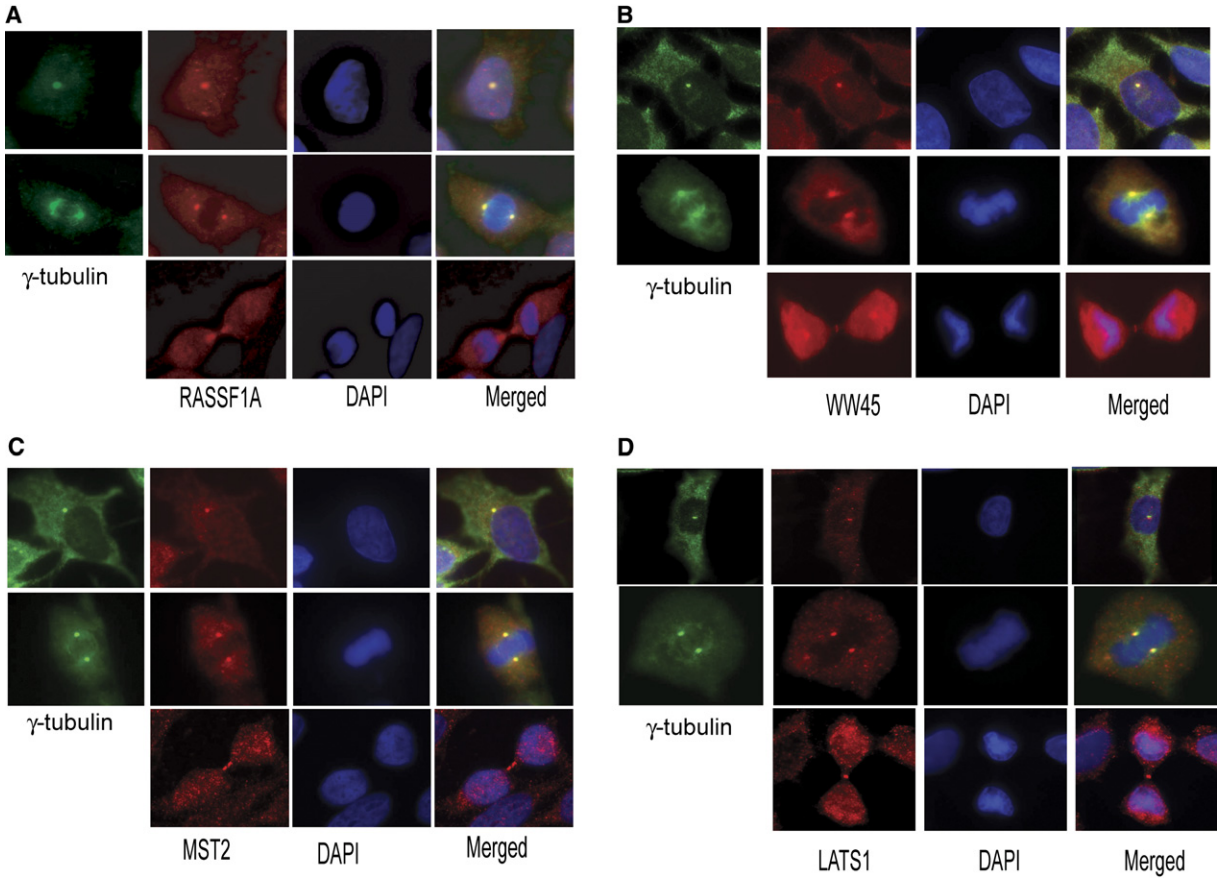

Figure 3. RASSF1A, MST2, WW45, and LATS1 Localize to the Centrosome and Mitotic Apparatus

HeLa cells were grown on coverslips in six-well plates, fixed, and stained with specific antibodies. Cells in interphase (top rows), metaphase (middle rows), and cytokinesis (bottom rows) are shown.

(A) Purified rabbit anti-RASSF1A and mouse anti-γ-tubulin were used for immunostaining.

(B) Rabbit anti-WW45 and mouse anti-γ-tubulin were used for coimmunostaining.

(C) Goat anti-MST2 was used for costaining with mouse anti-γ-tubulin.

(D) Cells were costained with rabbit anti-LATS1 and mouse anti-γ-tubulin.

time-lapse video microscopy, we monitored wild-type MEFs and Rassf1a$^{-/-}$ MEFs during mitotic cell division (Figure 4; see also supplemental movies). We observed that Rassf1a$^{-/-}$ MEFs required a significantly extended time period to traverse mitosis (Figure 4). A substantially increased fraction (40%) of Rassf1a$^{-/-}$ MEFs compared to wild-type MEFs (17%) did not complete mitosis as a result of cytokinesis failure (Figure 4). Binucleated cells formed in most cases. By scoring >2000 cells of each genotype at passage 3, we found that 17% of Rassf1a$^{-/-}$ MEFs (362 of 2134) but only 12.9% of Rassf1a$^{+/+}$ MEFs (317 of 2463) had two or more nuclei ($p < 0.001$, Chi square test) indicating that multinucleated cells arise in Rassf1a$^{-/-}$ MEFs as a result of defects in mitosis. Microscopy data obtained by DAPI and anti-tubulin staining and the counting of all mitotic cells indicated a mitotic index of 1.22% (41 of 3363 cells) for wild-type MEFs and 2.27% (27 of 1192 cells) for Rassf1a$^{-/-}$ MEFs ($p < 0.025$, Chi square test) consistent with a defect in mitosis.

The mitotic defect of Rassf1a$^{-/-}$ cells could be complemented, at least partially, with a lentiviral vector expressing GFP-RASSF1A, which reduced the number of failed mitoses and accelerated mitotic progression (Figure 4E). Importantly, GFP-WW45 complemented the defect almost equally well as RASSF1A, and MST2 also promoted mitotic progression in Rassf1a$^{-/-}$ cells relative to the GFP-only control. A construct containing LATS1 did not express efficiently and could not be tested. The data suggest that the mitotic defect in Rassf1a$^{-/-}$ cells can be complemented by components of the mammalian Hippo pathway.

The Mammalian Hippo Pathway

This is the first demonstration that the components of the Hippo pathway interact with each other in mammalian cells. Although we confirmed the existence of RASSF1A, MST2, WW45, LATS1 complexes, we cannot exclude the possibility that more than one complex exists. For example, one could hypothesize that there is a specific complex involved in apoptosis and that a different complex participates in mitotic exit. The situation is complicated by the fact that LATS1 also has been shown to induce apoptosis [18, 19] and by the fact that multiple family members exist (RASSF1 through RASSF6, MST1 and MST2, LATS1 and LATS2).

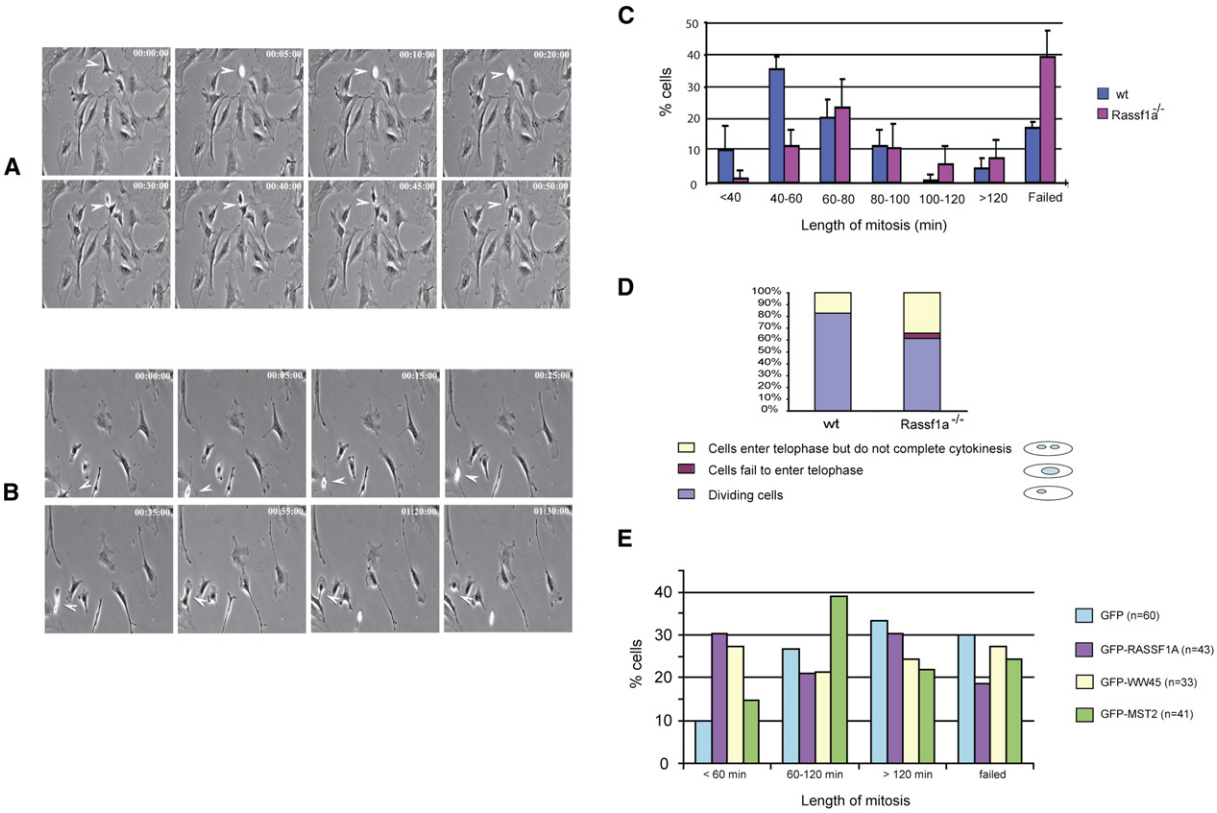

Figure 4. Cytokinesis Defect in Rassf1a-Knockout Cells

Wild-type and Rassf1a$^{-/-}$ MEFs were grown at passage 3 and were observed by time-lapse video microscopy.

(A) Mitosis in a Rassf1a$^{+/+}$ cell.

(B) Mitosis in a Rassf1a$^{-/-}$ cell. The white arrows point to the mitotic cells.

(C) Evaluation of the length of mitosis in a total of 112 Rassf1a$^{+/+}$ cells (blue) and 130 Rassf1a$^{-/-}$ cells (purple) derived from three independent embryos for each genotype (error bars, ± standard deviation).

(D) Diagram showing the outcome of mitosis in wild-type and Rassf1a$^{-/-}$ cells as determined by video microscopy.

(E) Complementation of the mitotic defect in Rassf1a$^{-/-}$ MEFs by lentiviral vectors expressing GFP-RASSF1A, GFP-WW45, or GFP-MST2. Video microscopy of the transduced cells was used for monitoring mitotic progression (the total number of cells recorded is shown in parentheses).

Mitosis was delayed and frequently resulted in cytokinesis failure in Rassf1a-KO cells (Figure 4). A similar phenotype has been observed in cells from Lats1-knockout mice or by RNA-mediated interference targeted against LATS1 [20, 21, 17]. Our data suggest that LATS1 is a downstream target of RASSF1A, WW45, and MST2, and inactivation of RASSF1A and LATS1 produce similar defects in mitotic progression. Cytokinesis failure can lead to the emergence of tetraploid cells, which are thought to be intermediates on a path leading to aneuploidy [22]. Lats1- and Rassf1-knockout mice are prone to tumors [3, 23]. Human *WW45* (Salvador) is deleted in several cancer cell lines [6]. *LATS1* and *LATS2* are downregulated by promoter methylation in about 50% of human breast cancers [24]. However, RASSF1A is probably the most commonly incapacitated component of this emerging tumor-suppressor pathway [2].

A tentative ortholog of the RASSF family of proteins can be identified in *Drosophila* (DmCG4656). In the C-terminal part (amino acids 190–340), RASSF1A is 28% identical and 45% similar to DmCG4656. However, this *Drosophila* gene product is more closely related to human RASSF2 and RASSF4. Recently, it has been shown that *Drosophila* Rassf inhibits the Hippo pathway [25], raising the important question of whether and how all six mammalian RASSF family members impinge on this pathway separately and in combination. The pathways are likely to be at least partially different in flies and humans. For example, mitotic defects have not been reported for fly cells with mutations in *hippo*, *sav*, or *lats*. The function of the mammalian complex in mitotic exit is similar to the function of some of these proteins in yeast, where Cdc15 (the Mst ortholog) activates Dbf2 (the Lats ortholog) in the mitotic-exit network [26]. However, there are no recognizable RASSF or WW45 orthologs in *S. cerevisiae*.

In summary, we demonstrated that RASSF1A is a component of a tumor-suppressor network similar to one described in *Drosophila*, which includes the mammalian homologs MST2, WW45, and LATS1. Defects in this pathway may lead to abnormal mitosis and failure to induce apoptosis. Further investigations are needed for dissecting the precise regulation of this complex in cell-cycle progression, apoptosis, and tumorigenesis.

Supplemental Data

Experimental Procedures, five figures, and six supplemental movies are available with this article online at http://www.current-biology.com/cgi/content/full/17/8/700/DC1/.

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