

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

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## 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*<sup>-/-</sup> 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*,

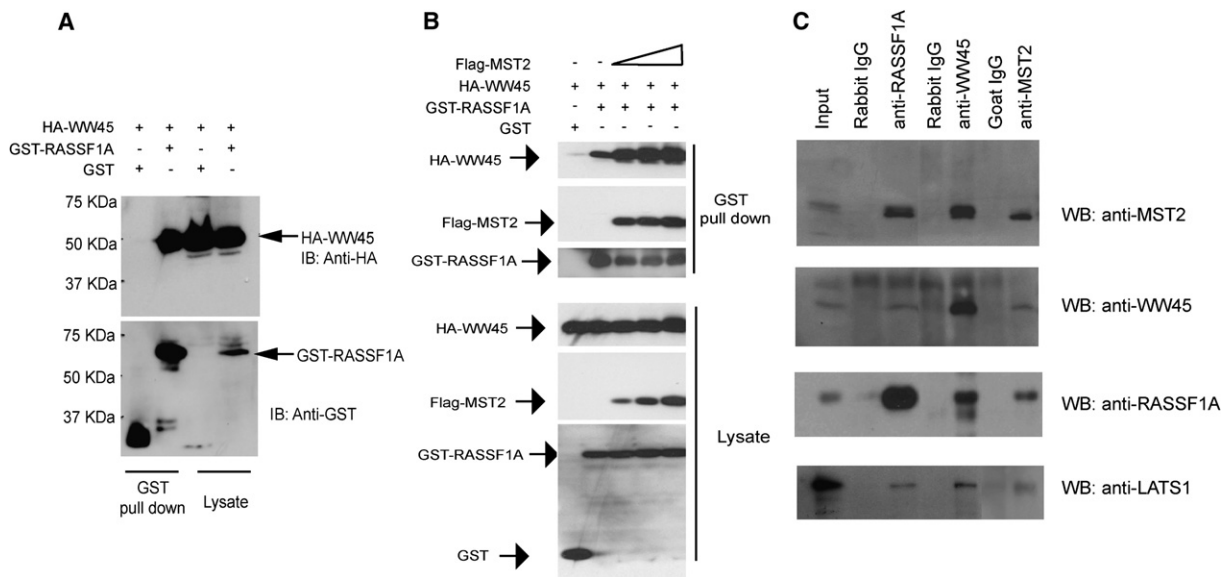
MST1, MST2, and Sav all contain a conserved C-terminal domain, which has been referred to as the SARAH (Salvador, *RASSF1*, Hippo) domain [11]. This domain is required for the interaction of *RASSF1A* with MST1 [4]; it may also be the module for interaction of the other proteins of the putative complex 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.

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**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  $\mu$ g), HA-WW45 (4  $\mu$ g), and different amounts (0.4, 0.8, and 2  $\mu$ g) 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

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