Salvador Protein Is a Tumor Suppressor Effector of RASSF1A with Hippo Pathway-independent Functions*§

Howard Donninger², Nadia Allen², Adrianna Henson², Jennifer Pogue‡, Andrew Williams‡, Laura Gordon‡, Susannah Kassler‡, Thomas Dunwell‡, Farida Latif¶, and Geoffrey J. Clark‡

From the ‡Department of Medicine, J. G. Brown Cancer Center, Molecular Targets Program, CTR Building, University of Louisville, Louisville, Kentucky 40202, §QIAGEN, Gaithersburg, Maryland 20878, and the ¶Section of Medical and Molecular Genetics, University of Birmingham, Edgbaston B15 2TT, United Kingdom

The RASSF1A tumor suppressor binds and activates proapoptotic MST kinases. The Salvador adaptor protein couples MST kinases to the LATS kinases to form the hippo pathway. Upon activation by RASSF1A, LATS1 phosphorylates the transcriptional regulator YAP, which binds to p73 and activates its proapoptotic effects. However, although serving as an adaptor for MST and LATS, Salvador can also bind RASSF1A. The functional role of the RASSF1A/Salvador interaction is unclear. Although Salvador is a novel tumor suppressor in Drosophila and mice, its role in human systems remains largely unknown. Here we show that Salvador promotes apoptosis in human cells and that Salvador inactivation deregulates the cell cycle and enhances the transformed phenotype. Moreover, we show that although the salvador gene is seldom mutated or epigenetically inactivated in human cancers, it is frequently down-regulated posttranscriptionally. Surprisingly, we also find that although RASSF1A requires the presence of Salvador for full apoptotic activity and to activate p73, this effect does not require a direct interaction of RASSF1A with MST kinases or the activation of the hippo pathway. Thus, we confirm a role for Salvador as a human tumor suppressor and RASSF1A effector and show that Salvador allows RASSF1A to modulate p73 independently of the hippo pathway.

Studies in Drosophila originally identified a novel tumor suppressor signaling pathway that involves the interaction of two tumor suppressor kinases, Hippo and Warts. The interaction of Hippo and Warts is mediated by an adaptor molecule called Salvador. Salvador binds both kinases, facilitating the phosphorylation and activation of Warts by Hippo (5–8). In Drosophila, Salvador was found to act as a tumor suppressor (9).

The hippo pathway is conserved in mammalian systems (8, 10, 11). The orthologs of Hippo and Warts are the proapoptotic MST kinases (12) and the proapoptotic LATS kinases (13). The human ortholog of Salvador is also referred to as Salvador. Both MST and LATS kinases exhibit properties compatible with a tumor suppressor function in mammalian systems, and LATS1 knockout mice develop tumors (13, 14). The pathways controlled by activated LATS are only now being revealed and appear to involve direct phosphorylation of the transcriptional coactivator YAP (15), which controls transcription factors such as the tumor suppressor p73 (16, 17). However, the function of YAP remains somewhat controversial, as it appears to be able to act as an oncogene or a tumor suppressor, depending upon the cellular context (18).

RASSF1A has been identified as a component of the MST/LATS pathway, as it binds MST kinases directly via a shared structural motif designated a SARAH² motif (19) and modulates p73 activation (20). However, the role of Salvador in this process may be complex because not only does Salvador bind and couple MST and LATS kinases, it can also form a direct complex with RASSF1A via the SARAH motif of RASSF1A (19). Thus, RASSF1A has the potential to feed into the hippo pathway at two different points.

Knockout mouse studies have implicated Salvador as a potential tumor suppressor in mammalian systems (21). However, the importance of Salvador in human tumorigenesis is unclear, as only two human tumor cell lines from a large panel have been identified with defects in the Salvador gene (9). Moreover, the contribution of Salvador to RASSF1A-mediated signaling and tumor suppression remains unknown.

To determine whether Salvador has the potential to serve as a tumor suppressor in human systems and if it may serve as a RASSF1A effector, we cloned the human form and examined its biological properties. We found that overexpression of Salvador induces apoptosis in human tumor cells and inhibits their...
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growth and survival. In contrast, inhibition of Salvador expression by shRNA deregulates the cell cycle and promotes the transformed phenotype in human cells. Moreover, we have found that the protein levels of Salvador are frequently down-regulated in tumor cells.

To address the role of Salvador in RASSF1A-mediated tumor suppression, we examined the ability of RASSF1A to stimulate p73 and apoptosis in a Salvador knockdown matched pair cell system. We found that Salvador was an essential component of the ability of RASSF1A to promote apoptosis and to stimulate p73. To understand why RASSF1A directly binds Salvador, we generated a point mutant of RASSF1A in the SARAH motif that is specifically defective for the interaction of the MST1 and MST2 kinases but retains essentially wild-type binding to Salvador. Surprisingly, although this mutant failed to activate the phosphorylation or nuclear translocation of YAP, it was not impaired for the ability to activate p73. However, in Salvador knockdown cells, both wild-type and mutant RASSF1A lost the ability to activate p73.

In conclusion, we show that Salvador is likely to be an important human tumor suppressor and effector of RASSF1A that is often inactivated in human tumor cells. We also show that the RASSF1A-Salvador complex can act independently of MST kinases, raising the possibility of hippo pathway-independent tumor suppressor functions for Salvador.

EXPERIMENTAL PROCEDURES

Plasmids—Human Salvador was cloned by PCR from an expressed sequence tag clone, confirmed by sequencing and cloned into pEGFP (Clontech, Mountain View, CA), FLAG-tagged pcDNA3.1 (22), and HA-tagged pBabe (23) as a BamH1/EcoR1 fragment. MST1 constructs were obtained from J. Chernoff (Fox Chase, Philadelphia, PA), and MST2 has been described previously (24). HA-RASSF1A has been described previously (25). pG13-Luciferase (26) was a generous gift of Dr. A. Zaika, Vanderbilt University. The L308P mutation of RASSF1A was engineered using a QuickChange kit (Stratagene, La Jolla, CA) and was based on the sequence of a partial cDNA sequence of a RASSF1A cDNA clone derived from a human tumor. Hush shRNAs to Salvador were purchased from OriGene (Rockville, MD), shRNA1 = TGCAGAAATTCCTGAC-3’ -ATACTGCAGAAATTCCTGAC-3’, shRNA2 = CCTGTGAAATTCTGAC-3’ -ATACTGCAGAAATTCCTGAC-3’.

Tissue Culture—Cell lines were grown in DMEM/10% FCS. Cells were transfected with Lipofectamine 2000 (Invitrogen) and, where necessary, selected in puromycin at 1 μg/ml. Caspase assays were performed by incorporating the pCaspase-3-Sensor expression plasmid (Clontech) into the transfection and quantifying nuclear localization of the caspase-cleaved GFP product after 24 h. For cell cycle analysis, MCF-7 cells were trypsinized, washed with PBS, counted for desired cell density, and then incubated for 10 min in NIM (10 mM Tris HCl, 0.1 mM EDTA, 0.5% Tween 20, 20 μg/ml RNaseA). After centrifugation at 4 °C for 10 min, cells were stained with 40 – 60 μM propidium iodide for a total of 30 min in the dark. Cells were acquired on a B-D Facsan utilizing a DNA program gating out doublets and analyzed on FLOWJO software.

Soft agar assays were performed as described previously (27). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assays were performed as recommended by the manufacturer of the Vybrant kit (Invitrogen).

Salvador Status in Tumors and Tumor Cell Lines—Salvador (SAV1) RT-PCR was performed on RNA extracted from cells before or after azacytidine treatment using primers 5’-ATACTGCAGAAATTCCTGAC-3’ (exon 4) and 5’-GCA-TTTATTAACCAGAAT-3’ (3’ UTR). GAPDH expression was used as a loading control, and semi-quantitative analysis of SAV expression was carried out as described previously (28, 29). Protein lysates from the indicated cell lines were run on SDS-PAGE, and Salvador protein expression was analyzed using our Salvador rabbit polyclonal antibody using Western blotting. A combination of kidney, breast, lung, colorectal, gliomas, and neuroblastoma cancer cell lines (n = 47) and kidney tumors (n = 48) were analyzed for mutation/deletions using direct sequencing of the five exons of the Salvador (hWW45) gene, as described previously (30). The 5’ CpG island of the hWW45 gene was analyzed for methylation, as described previously (30). Primer sequences are provided on request.

Protein Analysis and Antibodies—Exogenous expression of proteins was monitored by transient transfection of HEK-293-T cells followed by lysis and Western blotting analysis as described previously (31). Immunoprecipitations were performed using HA-conjugated-Sepharose beads (Sigma) according to the manufacturer’s instructions. Endogenous Salvador protein expression was visualized using a rabbit polyclonal against the hWW45 peptide EVSKPAEVQGKYVKKE by Open Biosystems (Huntsville, AL). The antibody was used at 1/500 dilution in Western blot analyses, which were visualized with an ECL kit. Phospho-YAP 127 antibodies were obtained from Cell Signaling Technology, Inc. (Danvers MA).

Luciferase Assays—Dual luciferase assays using a Renilla luciferase internal control were performed using the pG13-Luciferase reporter in MCF-7 cells with a Promega dual luciferase kit, essentially as described previously (32).

RESULTS

Salvador Inhibits Survival and Promotes Apoptosis in Human Tumor Cells—To determine the biological properties of Salvador in human cells, MCF-7 breast tumor cells were transfected with pBabe-Salvador and selected in puromycin. Surviving colonies were stained with crystal violet after 10 days. Overexpression of Salvador proved to be very growth inhibitory, and few colonies arose in the Salvador transfected plates compared with the empty vector (Fig. 1A). Those colonies that did eventually arise did not express detectable levels of exogenous Salvador (data not shown). To define the mechanisms behind the growth inhibition, we assayed the ability of Salvador to induce apoptosis in mammalian cells using a fluorescent indicator of caspase activation, pCaspase-3-Sensor (Clontech). This plasmid produces a GFP fusion protein that localizes to the nucleus/nucleolus when cleaved by caspase 3 but is otherwise cytoplasmic. MCF-7 cells were transfected with RFP-Salvador and pCaspase-3-sensor. Empty RFP vector served as the control. The percentage of cells expressing RFP that demonstrated activation of the Caspase-3-Sensor protein was determined by
Salvador knockdown cells were exhibiting enhanced proliferation rates (Fig. 2B).

**Down-regulation of Salvador Promotes the Transformed Phenotype**—To examine the effects of loss of Salvador function on the transformed phenotype, we plated the Salvador knockdown MCF-7 cell lines in soft agar and compared their ability to grow to that of the vector-transfected cells (Fig. 2D). The soft agar plates were examined after 10 days of growth. The shRNA-treated cells made more colonies, and the colonies that arose were larger.

**Salvador Is Frequently Down-regulated in Tumor Cells**—If Salvador serves as an important human tumor suppressor, then we might expect that it would be inactivated in human tumors at a significant frequency. Yet previously, deletions have been reported for Salvador in only two kidney tumor cell lines of more than 50 human tumor cell lines screened (9). We performed analyses of 48 primary kidney tumor samples for mutations, and 47 tumor cell lines (comprising kidney, breast, lung, colorectal, glioma, and neuroblastoma) for deletion or mutations and for promoter methylation of the Salvador gene. The only deletions found were in the two kidney cancer cell lines reported by Tapon et al. (9), and no significant evidence of promoter methylation was detected (data not shown). However, subsequent Western blot analysis of a series of kidney tumor cell lines showed that eight of 14 had lost or exhibited severely impaired expression of the Salvador protein (Fig. 3A). Further analysis of a subset of the cell lines showed that cells that had lost Salvador protein expression retained similar levels of mRNA expression as the cell lines that retained Salvador protein expression (Fig. 3B). Moreover, the mRNA levels of Salvador were unaffected by treatment with the demethylating drug 5-AzaC, ruling out epigenetic effects that were confirmed by our methylation analysis above. Thus, Salvador is frequently down-regulated in human tumor cells, but the mechanism appears to be posttranscriptional.

**RASSF1A and Salvador**

![Figure 1](image-url) **FIGURE 1.** Salvador inhibits growth and promotes cell cycle arrest and apoptosis. A, MCF-7 cells were transfected with 200 ng of pBabe vector or Salvador expression construct. Colonies were stained after selection in puromycin for 10 days. B, MCF-7 cells were transfected with pRFP Salvador or empty vector and pCaspase-3-sensor, which produces a fluorescent recombinant protein sensitive to caspase cleavage. Cells positive for caspase activation were scored. The results are the average of three separate assays.

counting under a fluorescent inverted microscope (as described previously (33)). Salvador enhanced the number of cells exhibiting caspase activation by a factor of approximately three (Fig. 1B).

**Down-regulation of Salvador Deregulates the Cell Cycle and Enhances Proliferation of Tumor Cells**—To determine the biological effects of down-regulating Salvador, we identified two effective Salvador shRNAs and generated stable Salvador knockdown cell lines. Knockdown was validated by Western blotting using our Salvador antibody (Fig. 2A). Salvador has been implicated in regulating cell cycle exit, and defects in the hippo/Salvador pathway cause a failure to arrest in G0/G1 in Drosophila (34). FACS analysis of the Salvador knockdown cell lines showed that the fraction of cells in G0/G1 during exponential growth of the cultures was significantly reduced (Fig. 2C), compatible with a failure to exit the cell cycle. Subsequent analysis of cellular proliferation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay confirmed that the Salvador knockdown cells were exhibiting enhanced proliferation rates (Fig. 2B).

**RASSF1A Forms a Complex with Salvador**—RASSF1A is a major human tumor suppressor that contains a SARAH motif and directly binds to MST kinases (1–3). However, it can also form an endogenous complex with Salvador (19). In Fig. 4A, we confirm this. RASSF1A also contains a Ras Association (RA) domain and forms a complex with activated K-Ras (2, 35). We have found previously that the RASSF1A effector MOAP-1 undergoes enhanced binding to RASSF1A in the presence of activated K-Ras (31). However, when we performed cotransfection/coimmunoprecipitation studies including K-Ras in the system, we found that there was no difference in the ability of Salvador to complex with RASSF1A (Fig. 4B). Thus, Salvador is likely to mediate Ras-independent functions of RASSF1A.

**Salvador Is Required for RASSF1A to Express Its Full Proapoptotic Activity**—To determine the contribution of Salvador to RASSF1A-induced apoptosis, we transfected the MCF-7 Salvador knockdown cell system with RASSF1A and measured the induction of caspase activity. The Salvador knockdown cells were resistant, although not completely, to RASSF1A-mediated caspase activation compared with the vector-transfected cells (Fig. 4C). This confirms Salvador as an important proapoptotic effector of RASSF1A. To determine whether Salvador functions downstream of RASSF1A, we established stable cells lines
derived from the H1299 lung cancer line (RASSF1A-negative) transfected with a RASSF1A expression vector or an empty vector (36). The ability of Salvador to induce caspase activation in this matched pair cell system was assayed (Fig. 4D). We detected no significant difference in the ability of Salvador to induce the apoptotic program in cells positive or negative for RASSF1A expression. This supports a role for Salvador downstream of RASSF1A.

RASSF1A Activates p73 in a Salvador-dependent Manner—Recently, RASSF1A has been shown to activate p73 via the hippo pathway (20). The proposed mechanism is that RASSF1A binds and activates MST2, which results in the activation of LATS, resulting in the phosphorylation and activation of the YAP transcriptional regulator. YAP then binds p73 and translocates it to the nucleus, enhancing its activity on proapoptotic genes such as PUMA. This scheme assumes that Salvador acts as a scaffolding molecule by binding both MST and LATS kinases, facilitating the phosphorylation of the latter by the former. Thus, we would anticipate that removing Salvador from

FIGURE 2. Down-regulation of Salvador promotes growth and transformation of MCF-7 cells. MCF-7 cells were stably transfected with two different Hush shRNAs against Salvador or scrambled control. A, Western blot analysis using our Salvador antibody confirmed the knockdown of Salvador. B, the resultant cell lines were assayed for effects on cell proliferation as measured by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. C, the cell lines were characterized for their cell cycle distribution during exponential growth relative to the vector control cell line. D, the stable MCF-7 Salvador knockdown cells were assayed for the ability to grow in soft agar. 5000 cells were plated, and the assay was scored after 10 days. Results are an average of two assays in duplicate. A representative experiment is shown in the right panel.

FIGURE 3. Salvador is frequently down-regulated in kidney tumor cell lines. A, samples from kidney cancer cell lines were assayed by immunoblotting using our Salvador antibody for levels of the 43-kD Salvador protein. Eight of 14 samples demonstrated complete or almost complete loss of Salvador expression. Tubulin was used as an internal loading control. B, examination of the mRNA levels of Salvador in several kidney tumor cell lines that are negative for protein expression showed little difference in the expression of Salvador mRNA (SAV1) compared with the Salvador protein expressing cell lines despite profound differences in the levels of Salvador protein. Treatment of the cell lines with the DNA methylation inhibitor AzaC did not alter the expression levels of the mRNA.
the system would inhibit the pathway. Fig. 5 shows that down-regulation of Salvador inhibits the ability of RASSF1A to activate a p73 luciferase reporter.

**Activation of p73 by RASSF1A Is Salvador-dependent but MST-independent**—It seemed reasonable to assume that the previous results simply confirmed that the established hippo pathway was reiterated in human cells and that RASSF1A was stimulating it by activating MST kinases and thence LATS kinases with Salvador serving to couple the activated MST to LATS. But this does not explain why RASSF1A can bind Salvador itself. In an attempt to dissect the role of the binding of RASSF1A to Salvador, in contrast to the binding to MST kinases, we identified a SARAH motif point mutant of RASSF1A (L308P) that was specifically defective for the interaction with the MST1 and 2 kinases. This mutant was based on a mutation identified in a tumor-derived RASSF1A cDNA sequence in the Genbank database. The mutant demonstrated no detectable binding to MST1 or MST2 (Fig. 6A), but it retained the wild-type ability to bind Salvador (Fig. 6B). This mutant failed to activate MST kinase activity (37) and is severely defective for stimulating the phosphorylation of YAP (Fig. 7A). Consequently, the mutant is also defective for inducing the nuclear localization of YAP and promoting the association of YAP with p73 (Fig. 7, B and C). However, we were surprised to find that the mutant was only modestly impaired for the ability to inhibit cell survival (Fig. 8A). Further analysis showed that the loss of binding of the L308P mutant to MST1 and MST2 had only a very small (but statistically significant) reduction in its ability to induce apoptosis (Fig. 8B) and no apparent effect on the ability of RASSF1A to modulate the cell cycle (C). Moreover, the L308P mutant retained the wild-type ability to activate the p73-dependent luciferase reporter (Fig. 8D). Critically, this activity remained dependent upon the presence of Salvador (Fig. 8D). Thus, the interaction between RASSF1A and Salvador allows RASSF1A to modulate p73 independently of the hippo pathway and supports hippo pathway-independent functions for Salvador.

**DISCUSSION**

Studies in *Drosophila* first identified Salvador as a likely tumor suppressor and defined its role as that of an adaptor, coupling the Hippo and Warts kinases. This hippo pathway was found to be
conserved in human systems, where MST kinases are coupled to LATS kinases by Salvador (5–8). In *Drosophila*, Salvador modulates both the cell cycle and apoptosis and plays an important role in determining organ size (15, 38). The human hippo pathway (MST/Salvador/LATS) appears to act via the phosphorylation of a protein called YAP by LATS (39). YAP acts, in part, via binding and regulating the transcription factor p73 (16, 17). However, YAP is a complex signaling molecule that can exhibit the properties of an oncogene or a tumor suppressor, depending upon the cellular context (18). Moreover, the descriptions of its mechanism of action remain somewhat contradictory.

MST kinases directly bind to the tumor suppressor RASSF1A (40), and RASSF1A was shown to activate the hippo pathway, resulting in the activation of p73 (20). MST kinases and Salvador interact with each other via a shared protein/protein interaction domain containing a SARAH motif (40, 41). Curiously, a similar motif was detected in RASSF1A and it has been shown that Salvador also appears to bind RASSF1A (19, 41). Although the biological role of the MST and LATS kinases has been relatively well studied, the role of Salvador in mammalian systems is less well understood. Knockout mouse studies now strongly support a tumor suppressor role for Salvador in mammalian systems.

**FIGURE 6.** RASSF1A L308P does not bind to MST kinases but retains Salvador binding. A, 293-T cells were cotransfected with HA-tagged RASSF1A and Myc-tagged MST1 (left panel) or HA-RASSF1A and FLAG-tagged MST2 (right panel). Cells were lysed and immunoprecipitated (IP) with HA and then immunoblotted (IB) with Myc or FLAG, respectively. B, HA-tagged RASSF1A and RASSF1A L308P were cotransfected into 293-T cells with FLAG-tagged Salvador and immunoprecipitated with HA before immunoblotting with FLAG.

**FIGURE 7.** RASSF1A L308P is defective for activation of YAP. A, MCF-7 cells were transfected with wild-type RASSF1A or the L308P point mutant of RASSF1A. Levels of phospho-YAP induced were quantified by phospho-YAP-specific antibody as a measure of hippo pathway activation. Relative phosphorylation is shown below. Results are an average of two assays, and a representative blot is shown above. B, RASSF1A L308P is defective for promoting the association of YAP with p73. 293-T cells were transfected with FLAG-p73, HA-RASSF1A, and RFP-YAP. Cells were immunoprecipitated (IP) with anti-FLAG and then Western blotted (IB) for YAP. C, RASSF1A L308P is defective for promoting the nuclear localization of YAP. Nuclear and cytoplasmic fractions were prepared from cells transfected with wild-type or the L308P mutant of RASSF1A and examined for YAP expression by Western blot analysis. TFIIH and p38 were used as markers of the nuclear and cytoplasmic fractions, respectively.
systems (21, 42). However, the role and relative importance of Salvador in RASSF1A function and in the development of cancer in humans is not well characterized.

Here we demonstrate in human systems that Salvador inactivation by shRNA results in the deregulation of the cell cycle and the enhancement of the tumorigenic phenotype. In knock-out mouse studies, loss of Salvador induced up-regulation of YAP, and a YAP dominant negative could block some of the biological effects of Salvador loss (21, 42). The authors concluded that deregulation of YAP was at least partially responsible for the 

salvador

knockout phenotype. We have observed up-regulation of YAP in the MCF-7 Salvador knockdown cells (data not shown), and so it seems likely that YAP deregulation is also playing a role here.

Functional Salvador appears to be important for RASSF1A to manifest its full apoptotic activity, as knockdown of Salvador reduces the ability of RASSF1A to induce apoptosis. This effect was at least partially specific, as the Salvador knockdown cells were not resistant to staurosporine, an activator of the intrinsic apoptotic pathway (43) (supplemental Fig. S1).

Thus, Salvador appears to be a potential human tumor suppressor and a proapoptotic effector of RASSF1A. The K-Ras oncoprotein can mediate apoptosis by forming a complex with RASSF1A. This interaction enhances the binding of RASSF1A to its proapoptotic effector MOAP-1 (31). Examination of the effects of K-Ras on the interaction of Salvador and RASSF1A showed that the interaction was not affected by activated K-Ras. This suggests that the RASSF1A/Salvador pathway is acting independently of Ras.

If Salvador is a 

bona fide

tumor suppressor, then we might expect to find that it becomes inactivated by some mechanism at a significant frequency in human tumor cells. Previously, a screen of 50 cell lines confirmed two that exhibited deletions in Salvador (9). In our screen, we confirmed the deletions found in the MCF-7, T47D, and MDA-MB-231 cell lines.

**FIGURE 8. RASSF1A L308P retains growth inhibitory activity and retains a Salvador-dependent ability to activate p73.** A, MCF-7 cells were transfected with 500 ng of wild-type or L308P mutant of RASSF1A in the vector pBabe. Cells were selected in puromycin for 2 weeks and then fixed and stained. B, the ability of wild-type and L308P mutant forms of RASSF1A to induce caspase activation were compared as described in the legend to Fig. 1. C, the effects of the wild-type and L308P forms of RASSF1A were compared for their effects on the cell cycle. D, MCF-7 cell lines transfected with vector or two different shRNAs to Salvador were transfected with 500 ng of the wild-type or L308P mutant of RASSF1A and 100 ng of a p73-luciferase reporter. Renilla luciferase was included as an internal control. Dual luciferase assays were performed on the transfected cells. Results are the average of three assays performed in duplicate.
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the original report (9), and no further mutations were identified in a panel of 47 cancer cell lines and 48 primary kidney tumors. Moreover, there was little evidence of epigenetic inactivation. However, when we examined the levels of Salvador protein present in a panel of kidney tumor cell lines, we found that the protein was often absent, despite the presence of mRNA. This suggests a posttranscriptional mechanism of inactivation. Experiments were performed to examine the effects of protease-some inhibitors and Calpain inhibitors on the expression of Salvador in the cell lines negative for Salvador protein expression. These treatments failed to rescue Salvador expression (data not shown). Moreover, incubation of Salvador-transfected HEK-293-T cell lysates with lysates from Salvador-negative tumor cell lines failed to induce differential degradation of the Salvador protein (data not shown). Thus, the mechanism for the frequent inactivation of Salvador protein expression may be at the translational level rather than at the protein stability level. This type of inactivation of tumor suppressors is not uncommon (44).

RASSF1A has been implicated as an activator of the p73 tumor suppressor via the MST/LATS pathway (20). In this scenario, it is proposed that RASSF1A-induced activation of LATS1 causes the phosphorylation of YAP on serine 127 and its subsequent release from LATS1 in the cytoplasm so that it can bind and activate p73 in the nucleus. Salvador acts as a linker, connecting MST kinases to the LATS kinases (45), and RASSF1A/MST2-induced phosphorylation of LATS1 is strongly enhanced by adding exogenous Salvador to the system (19). Thus, we can easily imagine a RASSF1A/MST/Salvador/ LATS/YAP pathway leading to the activation of p73. Yet the situation may be more complex, as RASSF1A appears to be able to bind Salvador directly (19). Moreover, it has been reported that phosphorylation of YAP on serine 127 promotes its binding to 14-3-3 proteins, leading to cytoplasmic retention and inactivation of p73, not activation. Thus, there appears to be something of a paradox in explaining the actions of RASSF1A on YAP/p73. In an attempt to resolve some of these issues, we developed a point mutant of RASSF1A (L308P) that is completely defective for the interaction with MST1 and MST2 but retains a wild-type association with Salvador (Fig. 6). This mutant demonstrated a modest reduction in the ability to kill cells (Fig. 8A) but lost all ability to activate MST kinase activity (37) and almost all ability to induce YAP phosphorylation, nuclear localization, or binding to p73 (Fig. 7). It also lost the ability to stabilize the overall levels of YAP, which has previously been reported to be a consequence on p73-YAP complex formation (17). However, we were surprised to find that the L308P mutant of RASSF1A retained the wild-type ability to activate a p73 luciferase reporter (Fig. 8B). This activity remained dependent upon the presence of Salvador. Thus, although RASSF1A does appear to be able to stimulate YAP phosphorylation by the hippo pathway, the activation of p73 by RASSF1A can be independent of the canonical hippo pathway. Consequently, Salvador may have functions outside of coupling MST to LATS. These results add a further layer of complexity to the regulation of the hippo pathway and may help resolve an apparent contradiction in our understanding of the relationship between RASSF1A and p73.

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