

Proapoptotic Kinase MST2 Coordinates Signaling Crosstalk between RASSF1A, Raf-1, and Akt

David Romano¹, David Matallanas¹, Gregory Weitsman³, Christian Preisinger¹, Tony Ng³, and Walter Kolch^{1,2}

Abstract

Mammalian MST kinases function in stress-induced apoptosis to limit tumor progression. However, there is limited understanding about MST2 control by key regulators of cell division and survival. Raf-1 binds and inhibits MST2 kinase, whereas dissociation from Raf-1 and binding to tumor suppressor protein RASSF1A activates MST2. Akt phosphorylates MST2 in response to mitogens, oncogenic Ras, or depletion of tumor suppressor phosphatase and tensin homologue deleted on chromosome 10. We identified T117 and T384 as Akt phosphorylation sites in MST2. Mutation of these sites inhibited MST2 binding to Raf-1 kinase but enhanced binding to tumor suppressor RASSF1A, accentuating downstream c-Jun NH₂-terminal kinase and p38 mitogen-activated protein kinase signaling and promoting apoptosis. We determined that MST2 phosphorylation by Akt limits MST2 activity in two ways: first, by blocking its binding to RASSF1A and by promoting its association into the Raf-1 inhibitory complex, and second, by preventing homodimerization of MST2, which is needed for its activation. Dissociation of the Raf-1–MST2 complex promoted mitogenic signaling and coordinately licensed apoptotic risk. Using Ras effector domain mutants, we found that Akt is essential to prevent MST2 activation after mitogenic stimulation. Our findings elucidate how MST2 serves as a hub to integrate biological outputs of the Raf-1 and Akt pathways. *Cancer Res*; 70(3): 1195–203. ©2010 AACR.

Introduction

Apoptosis is a safeguard against unlicensed growth of aberrant cells. Thus, proapoptotic signaling pathways are often silenced in tumors. MST1 and MST2 are involved in proapoptotic signaling in response to stress signals (1). MST1 induces c-Jun NH₂-terminal kinase (JNK) and caspase activation in response to stress and Fas (2, 3). During apoptosis, MST kinases are cleaved by caspases, releasing a constitutively active kinase domain that translocates to the nucleus and phosphorylates histone 2B, eventually inducing DNA fragmentation (4). In neuronal cells, MST1 mediates oxidative stress-induced apoptosis by phosphorylating and activating FOXO3 transcription factors (5). In cancer cells, MST2 is activated by RASSF1A and mediates apoptosis via induction of

the proapoptotic BH3 family gene *puma* (6) and activation of the Bax binding protein MAP-1/MOAP-1 (7, 8). Thus, in mammalian cells, MST kinases are bona fide apoptosis inducers in response to stress and tumor suppressor genes.

RASSF1A is a prominent tumor suppressor that is frequently inactivated in many different cancers usually by gene silencing (9–11). Binding of MST2 by RASSF1A induces MST2 activation by promoting dimerization and transphosphorylation of T180. The Raf-1 proto-oncogene binds to MST2, thereby inhibiting RASSF1A association and dimerization (12). Downregulation of Raf-1 can substitute for the loss of RASSF1A expression (6), suggesting that Raf-1 binding to MST2 is critical for preventing RASSF1A-mediated MST2 activation and apoptosis. However, mitogenic stimulation and activated Ras also can disrupt the MST2–Raf-1 complex without causing MST2 activation (13, 14). This suggests that MST2 release is part of normal mitogenic regulation and that mitogens must suppress the proapoptotic function of MST2.

Recent work showed that Akt phosphorylates MST1 on S387, blocking MST1 cleavage by caspases and its ability to phosphorylate FOXO3 (15). Akt is a main downstream effector of phosphoinositide 3-kinase (PI3K), whose pleiotropic functions include the inhibition of apoptosis (16–18). The Akt pathway is often deregulated in cancer due to mutations in Akt or p110 PI3K or more commonly because of mutations, silencing, or deletion of PTEN (19). PTEN is a tumor suppressor that dephosphorylates phosphatidylinositol 3,4,5-triphosphate, the product of PI3K (20). Here, we show that MST2 is phosphorylated by Akt, which inhibits its proapoptotic activity, but by a completely different mechanism than MST1. Akt-induced phosphorylation of MST2 (*a*) promotes

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MST2 interaction with Raf-1, (b) blocks MST2 recruitment by RASSF1A, and (c) directly inhibits MST2 kinase activity.

Materials and Methods

Cells, reagents, and plasmids. MCF7 and HeLa cells were grown in DMEM, and HCC1937 cells were grown in RPMI 1640, supplemented with 10% FCS (Life Technologies) and 2 mmol/L L-glutamine (Life Technologies). Human recombinant insulin-like growth factor-I (IGF-I) was from PromoCell. Cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. LY294002 and Akt inhibitor IV were from Calbiochem (Merck). Protein G-Sepharose and anti-Flag-M2 agarose conjugated were from Sigma. Antibodies were from commercial sources: mouse monoclonal anti-Raf-1 (BD Transduction Laboratories); mouse monoclonal anti-phospho-JNK (Ser⁷³; Upstate); goat polyclonal anti-Krs1/MST2, mouse monoclonal anti-PTEN A2B1, mouse monoclonal anti-RASSF1 3F3, and rabbit polyclonal anti-JNK-1 C19 (Santa Cruz Biotechnology); rabbit polyclonal anti-phospho-p38 and total p38, anti-phospho-Akt (Ser⁴⁷³) and total Akt, anti-phospho-glycogen synthase kinase 3 β (GSK3 β ; Ser²¹) and total GSK3 β , anti-phosphoserine/phosphothreonine substrate of Akt, and anti-phosphoserine-14-3-3 binding motif (Cell Signaling, New England Biolabs); anti-HA-HRP3F10 (Roche); anti-Flag-M2-HRP and mouse monoclonal anti-phospho-extracellular signal-regulated kinase (ERK) 1/2 and total ERK1/2 (Sigma); rabbit monoclonal anti-MST2 (Epitomics, Insight Biotechnology); and mouse monoclonal anti-RASSF1A (eBioscience). Scramble (control), Raf-1, MST2, and Akt1/2 small interfering RNAs (siRNA) were from Dharmacon and described previously (6). pcDNA3.1-HA-RASSF1A and pSG5-gag-Akt were previously described (21). pcDNA3.1-PTEN and pcDNA3.1-PTENC124S were a kind gift from Nick Leslie (CLS, University of Dundee, Dundee, United Kingdom). pCEFL-AU5-HRasV12 and the domain-specific mutants (S35 and C40) were generously provided by Piero Crespo (IDICAN, University of Cantabria, Santander, Spain). pME18S-Flag-MST2 (12) was used to make point mutants using the Quik-Change kit (Stratagene). Myc-MST2 was a kind gift from J. Chernoff (Fox Chase Cancer Center, Philadelphia, PA). MST2 wild-type and T117/384AA double mutant were cloned into pEGFP-C3 (Clontech) to make MST2-GFP. Raf-1 was cloned into pcDNA3.1 (Invitrogen) that was modified by the addition of mRFP1 to make mRFP1-Raf-1.

Coimmunoprecipitations, immunoblottings, and in-gel kinase assays. Cells were serum starved overnight and lysed in 20 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1% NP40, 2 mmol/L NaF, 10 mmol/L β -glycerophosphate, 2 mmol/L Na₂P₂O₄, and protease and phosphatase inhibitors. Immunoprecipitates were washed thrice in lysis buffer, separated by SDS-PAGE, and analyzed by Western blotting. Alternatively, in-gel kinase assays were performed using MST2 immunoprecipitates as previously described (12).

FRET determination by multiphoton fluorescence lifetime imaging microscopy measurements. Time-domain fluorescence lifetime imaging microscopy (FLIM) and analysis of the data were performed as described previously (22).

Apoptosis assays. Attached and floating cells were harvested, washed with PBS, resuspended in 0.25% trypsin, fixed in 70% ethanol, incubated with RNase (250 μ g/mL; Qiagen), and stained with propidium iodide (10 μ g/mL; Sigma) before analysis with a FACSCalibur flow cytometer (Becton Dickinson) to determine DNA fragmentation shown as fold increase in the sub-G₁ population.

In vitro kinase and binding assays. Purified Akt and MST2 (100 ng; ProQinase) were incubated for 5 min in 100 μ L of kinase buffer [50 mmol/L Tris-HCl (pH 7.4), 0.1 mmol/L EGTA, 10 mmol/L MgCl₂, 0.1% β -mercaptoethanol \pm 0.5 mmol/L ATP]. Each kinase reaction (2 μ L) was Western blotted with the anti-phosphoserine/threonine substrate of Akt antibody to assess phosphorylation. The remainder was split into two aliquots and incubated with RASSF1A or Raf-1 immunoprecipitates, washed, and analyzed by Western blotting with the anti-phosphoserine/threonine substrate, MST2, and Akt antibodies. The immunoprecipitates were prepared from HEK293 cells transfected with Flag-tagged Raf-1 or RASSF1A. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer, and anti-Flag-M2 agarose immunoprecipitates were washed five times in RIPA buffer and once in kinase buffer.

Results

MST2-Raf-1 interaction is promoted by PI3K/Akt. We examined whether the PI3K/Akt pathway regulates the MST2-Raf-1 complex. LY294002, a PI3K inhibitor, induced a time-dependent decrease of Raf-1 coimmunoprecipitation with MST2 and vice versa (Fig. 1A; Supplementary Fig. S1A). As observed previously (12), serum also decreased Raf-1 and MST2 coimmunoprecipitations but with a lower efficiency and a longer delay than LY294002 treatment. LY294002 enhanced and accelerated the serum-induced disruption of MST2-Raf-1 (Fig. 1A; Supplementary Fig. S1A). These results were confirmed using AktI, a specific Akt inhibitor (Fig. 1B; Supplementary Fig. S1B). The efficacy of the inhibitors was ascertained by showing that LY294002 blocked Akt phosphorylation (Fig. 1A), and AktI inhibited phosphorylation of the Akt substrate GSK3 (Fig. 1B). Importantly, LY294002 increased MST2 kinase activity both in untreated and serum-stimulated cells (Fig. 1A; Supplementary Fig. S1A). Treatment with IGF-I produced similar results (Supplementary Fig. S1C), suggesting that the PI3K/Akt pathway promotes the MST2-Raf-1 interaction and negatively regulates MST2 activity.

MST2 is a substrate for Akt. Serum induced coimmunoprecipitation of Akt with MST2 (Fig. 2A; Supplementary Fig. S2A), which was reduced by LY294002. An antibody specific for Akt substrate phosphorylation motifs revealed a PI3K-dependent increase in Akt-induced phosphorylation of MST2 in immunoprecipitates (Fig. 2A; Supplementary Fig. S2A). Overexpression of a constitutively active form of Akt, gag-Akt, induced a concomitant increase of coimmunoprecipitated Akt with MST2 and hyperphosphorylation of MST2 on Akt motifs. In addition, Raf-1 coimmunoprecipitated with MST2 increased, whereas MST2 kinase activity decreased proportionally (Fig. 2B; Supplementary Fig. S2B). Reexpression of RASSF1A counteracted the Akt effects, decreasing both the coimmunoprecipitation of

Akt with MST2 and phosphorylation of MST2 while elevating MST2 kinase activity (Fig. 2B; Supplementary Fig. S2B).

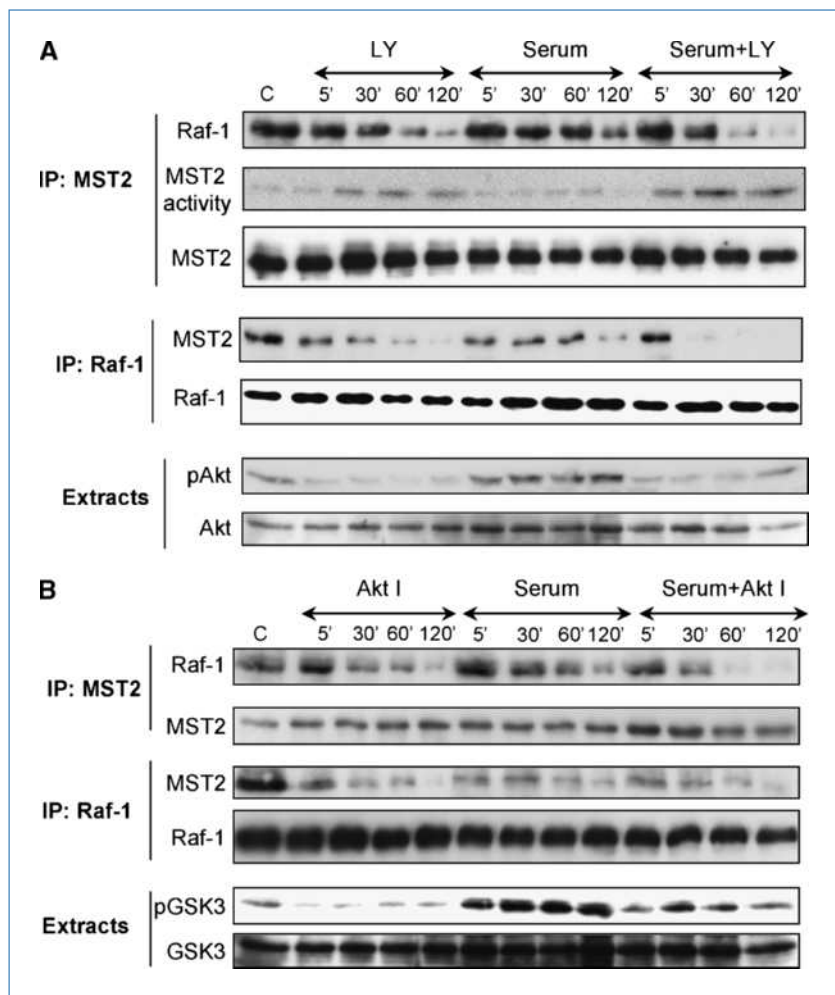
Akt inhibits MST2 by a dual mechanism. To clarify whether Akt phosphorylation inhibits MST2 kinase activation only by promoting Raf-1 binding or also directly, we used H-RasV12 effector domain mutants (Fig. 2C). H-RasV12S35 and H-RasV12C40 selectively stimulate Raf-1 and PI3K, respectively (23). Serum stimulation and expression of H-RasV12 or H-RasV12S35 decreased Raf-1 coimmunoprecipitated with MST2. In contrast, serum stimulation, H-RasV12, and the specific PI3K activator mutant H-RasV12C40 increased both Akt coimmunoprecipitation with MST2 and Akt-induced phosphorylation of MST2. Importantly, serum stimulation, H-RasV12, and H-RasV12C40, but not H-RasV12S35, inhibited RASSF1A binding to MST2 and MST2 kinase activity. By contrast, H-RasV12S35 disrupted the MST2–Raf-1 complex and increased RASSF1A recruitment and MST2 kinase activity. These results indicate that Akt exerts dual control over MST2 by preventing binding to RASSF1A while promoting interaction with Raf-1 and by directly inhibiting MST2 activity. Both processes require Akt kinase activity.

Akt regulates MST2 in cancer cells. PTEN loss is frequent in cancer, resulting in the hyperactivation of PI3K/Akt signaling (24). In the *PTEN*^{-/-} breast cancer cell line HCC1937, endogenous Akt was highly activated (Fig. 3A). Despite the retention of RASSF1A expression, MST2 had little kinase activity, efficiently coimmunoprecipitated with Raf-1, and was phosphorylated on Akt consensus sites. Reexpression of wild-type PTEN reversed all these parameters, whereas the phosphatase-dead C124S mutant was without effect (Fig. 3B; Supplementary Fig. S3A).

As Akt can bind Raf-1 (25, 26), we tested whether Raf-1 promotes the phosphorylation of MST2 by Akt. Raf-1 downregulation increased the MST2 and RASSF1A interaction and MST2 kinase activity even in the absence of a functional PTEN. Interestingly, Raf-1 downregulation only slightly decreased MST2 phosphorylation by Akt (Fig. 3B; Supplementary Fig. S3A), suggesting that Raf-1 is not required for the Akt-induced phosphorylation of MST2.

However, Akt is essential, as Akt downregulation by siRNA completely reversed the effects of PTEN loss, decreasing MST2–Raf-1 association and Akt phosphorylation of MST2

Figure 1. PI3K/Akt regulates MST2–Raf-1 interaction and MST2 activity. Serum-starved HeLa cells (C) were treated with LY294002 (LY; 5 μ mol/L; A) or Akt inhibitor (AktI; 10 μ mol/L; B) in the presence or absence of 10% serum (Serum+LY or Serum+AktI) for the indicated periods of time. In the serum + LY294002 and serum + AktI conditions, cells were preincubated for 30 min with LY294002 or AktI, respectively, before stimulation with 10% serum. MST2 or Raf-1 immunoprecipitates (IP) were analyzed by Western blots using antibodies for the indicated proteins. MST2 kinase activity was determined by in-gel kinase assays as described in Materials and Methods. Cellular extracts (10 μ g) were analyzed by Western blotting using phospho-Akt (Ser⁴⁷³) or phospho-GSK3 β (Ser²¹) antibodies and then reprobbed with antibodies against total Akt and total GSK3 β , respectively.



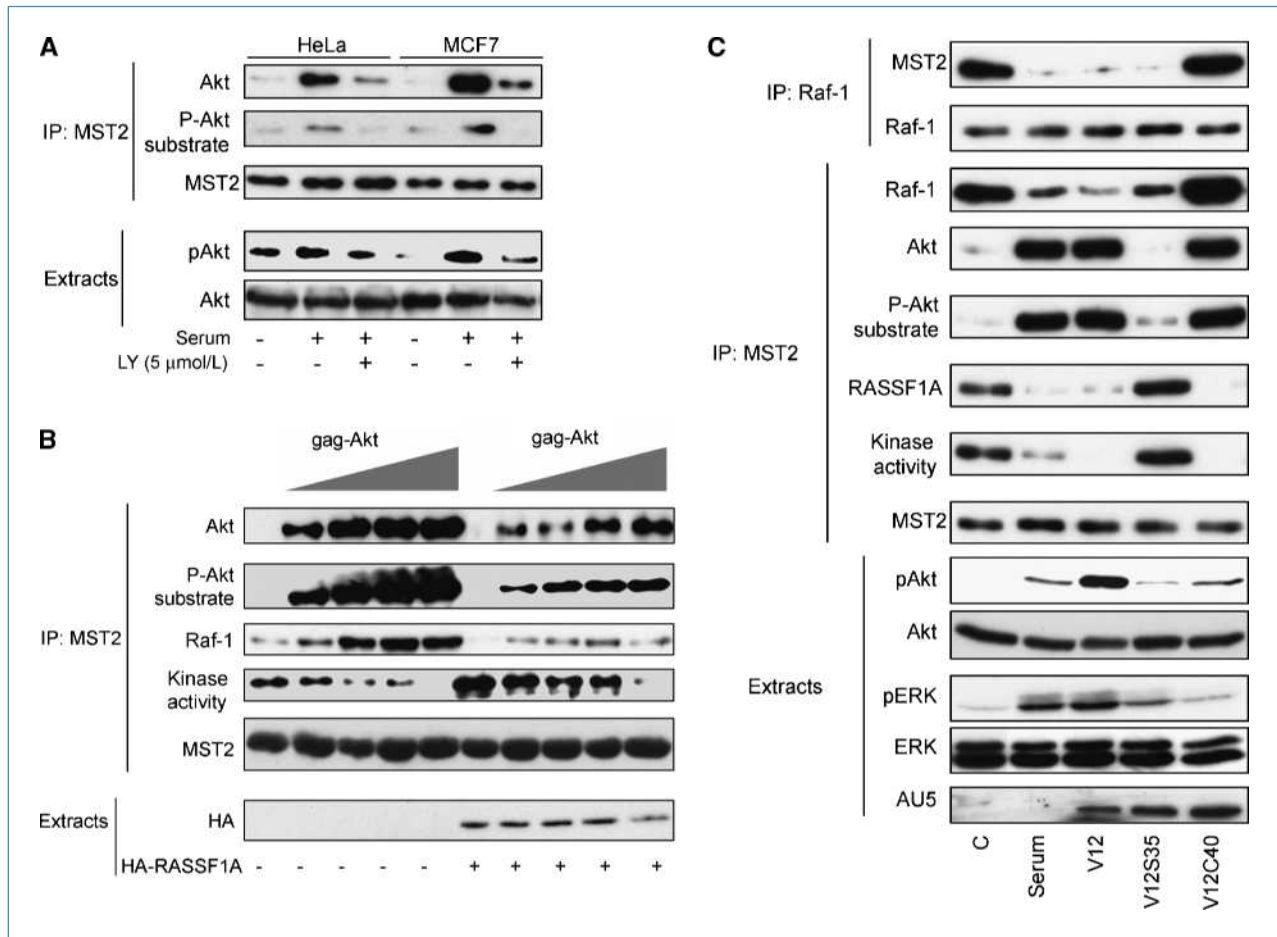


Figure 2. MST2 interacts with Akt and is a target for Akt-induced phosphorylation. **A**, serum-starved HeLa and MCF7 cells were treated with 10% serum for 2 h in the presence or absence of LY294002 (preincubated for 30 min). MST2 immunoprecipitates were analyzed by Western blotting using phosphoserine/phosphothreonine substrate of Akt (*P-Akt substrate*) and total Akt antibodies. Cellular extracts (10 μ g) were Western blotted using a phospho-Akt (Ser⁴⁷³) antibody and then reprobbed with a total Akt antibody. **B**, MCF7 cells were cotransfected with increasing amounts of gag-Akt (0, 0.1, 0.25, 0.5, and 1 μ g) and HA-RASSF1A where indicated. MST2 immunoprecipitates were analyzed by Western blotting with the indicated antibodies. MST2 kinase activity was measured by in-gel kinase assays. Cellular extracts (10 μ g) were analyzed by Western blot using HA antibody. **C**, HeLa cells were transfected with H-RasV12 (V12), H-RasV12S35 (V12S35), or H-RasV12C40 (V12C40). Raf-1 and MST2 immunoprecipitates were analyzed by Western blot using antibodies against the indicated proteins.

while increasing MST2 kinase activity. In addition, RASSF1A now could engage MST2 (Fig. 3C).

We then tested whether Akt affected MST2 homodimerization, an early event required for MST2 activation, by assessing coimmunoprecipitation of Flag- and Myc-tagged MST2. In *PTEN*^{-/-} HCC1937 cells, reexpression of wild-type PTEN increased MST2 homodimerization, whereas PTEN C124S had no effect (Fig. 3D). In MCF7 cells, the expression of gag-Akt decreased MST2 homodimerization, and this effect was counteracted by reexpressing RASSF1A (Supplementary Fig. S3B).

Taken together, these data suggest that Akt is a physiologic regulator of MST2 that cooperates with Raf-1 to prevent MST homodimerization and activation in cancer cells.

Akt induces MST2 phosphorylation on Thr¹¹⁷ and Thr³⁸⁴. MST2 contains two Akt consensus phosphorylation motifs: T117 and T384 (Supplementary Fig. S4A). To assess

the role of these sites, we generated single (T117A and T384A) and double (T117/384AA) Flag-tagged MST2 mutants. When expressed in *PTEN*^{-/-} HCC1937 cells, both single mutants showed reduced interaction with Raf-1 and Akt and reduced phosphorylation on Akt consensus sites (Fig. 4A). The double T117/384AA mutant was completely devoid of binding to Raf-1 or Akt and Akt phosphorylation. MST2 single mutants coimmunoprecipitated more efficiently with RASSF1A and had enhanced kinase activity, which was further enhanced by the double mutation. PTEN expression increased the interaction of RASSF1A with MST2 and MST kinase activity, whereas the MST2 mutants were largely unaffected. Mutation of the activating autophosphorylation site T180 had no effect on any of these parameters (Fig. 4A; Supplementary Fig. S4B). These results suggest that Akt phosphorylation of MST2 at T117 and T384 inhibits binding to RASSF1A and concomitant MST2 activation while facilitating

binding to Raf-1. Therefore, PTEN loss contributes to the shutdown of MST2 signaling in cancer cells.

Does the Akt-mediated regulation of MST2 depend on Raf-1? Downregulation of Raf-1 in *PTEN*^{-/-} cells only slightly affected the kinase activities of wild-type and mutant MST2 (Fig. 4B; Supplementary Fig. S4C), suggesting that the phosphorylation by Akt exerts a dual effect on MST2. First, it prevents RASSF1A binding and promotes sequestration of MST2 into the inhibitory complex with Raf-1. Second, it has a direct inhibitory effect on the catalytic activity of MST2, which does not require MST2 interaction with Raf-1. Thus, these data independently confirm the results from the Ras effector domain mutant experiments.

To confirm the biochemical interactions by *in situ* imaging, we performed FLIM. GFP-MST2 and mRFP1-Raf-1 expressed in HCC1937 cells interacted as indicated by a shortening of FLI in comparison with GFP-MST2 alone, and this interaction was significantly decreased when PI3K was inhibited with LY294002 (Fig. 4C). Mutation of the two Akt phosphorylation sites (GFP-MST2-TT117/384AA) inhibited the interaction with Raf-1 (Fig. 4D), confirming the biochemical observations that Akt-induced phosphorylation of MST2 is critical for the MST2–Raf-1 interaction.

Akt phosphorylates MST2 directly and inhibits binding to RASSF1A. To assess the distribution of MST2 between the two competing binding partners, we analyzed Raf-1 and RASSF1A immunoprecipitates for the presence and phosphorylation status of MST2 in HCC1937 cells (Fig. 5A). Wild-type MST2 preferentially coimmunoprecipitated with Raf-1. PTEN

expression diminished MST2 association with Raf-1 but enhanced binding to RASSF1A. Whereas MST2 was phosphorylated on the Akt sites in Raf-1 immunoprecipitates, RASSF1A immunoprecipitates were devoid of phosphorylated MST2. By contrast, MST2-TT117/384AA showed the opposite behavior, readily associating with RASSF1A but not with Raf-1.

In vitro kinase assays with purified Akt and MST2 proteins showed that MST2 was a direct Akt substrate, although some background phosphorylation of the Akt sites was observable when MST2 was incubated with ATP alone (Fig. 5B). The *in vitro* kinase reactions were subsequently incubated with Raf-1 or RASSF1A immobilized on beads (Fig. 5C and D). Phosphorylated MST2 bound to Raf-1 but not to RASSF1A. Interestingly, MST2 binding to Raf-1 was enhanced by the presence of Akt, raising the possibility that Akt can facilitate this interaction. However, in cells, Akt can be recruited to MST2, whereas Raf-1 dissociates (Fig. 2C), suggesting that this adaptor role of Akt is further regulated in cells or an *in vitro* artifact. However, RASSF1A consistently only bound nonphosphorylated MST2 *in vitro* and in cells.

These data suggest that MST2 is a direct Akt substrate and that this phosphorylation precludes MST2 binding to RASSF1A but enhances binding to Raf-1.

Akt regulates MST2 proapoptotic function. Inhibiting MST2 expression by siRNA in HeLa cells reduced both LY294002-induced and AktI inhibitor-induced apoptosis, as assayed by DNA fragmentation, to a modest but significant extent (Fig. 6A and B). To examine the role of Akt-mediated MST2 phosphorylation, we overexpressed MST2 and the

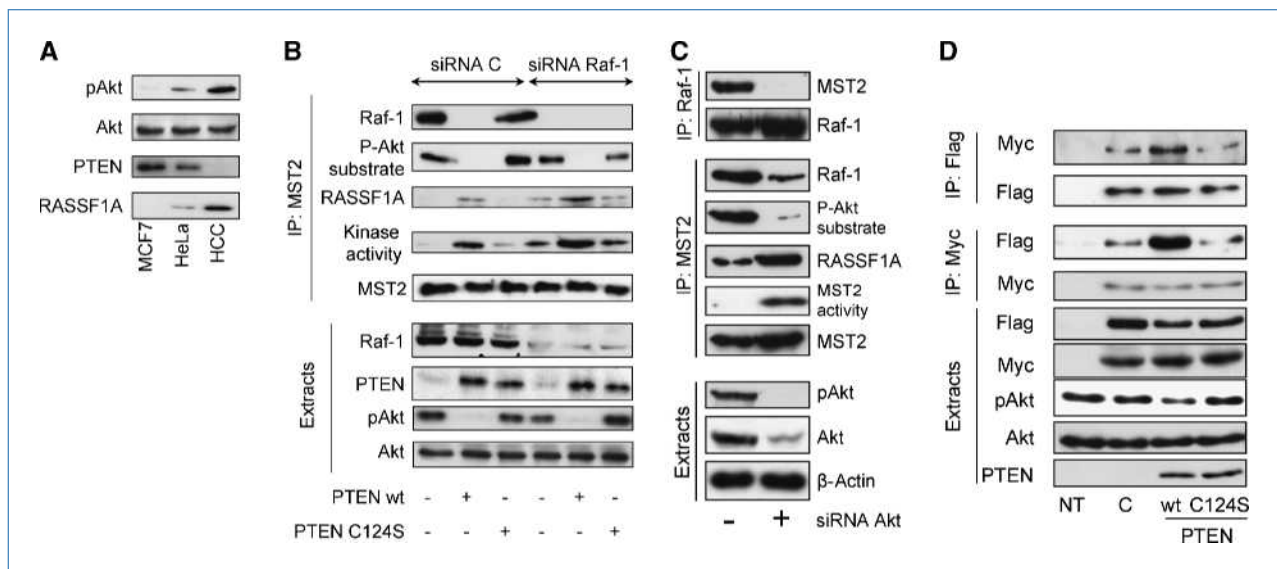


Figure 3. Regulation of endogenous MST2 activation by Akt, PTEN, and Raf-1. A, 10 μ g of cellular extracts from MCF7, HeLa, and HCC1937 cells were analyzed for Akt activation [phospho-Akt (Ser^{A73})] and expression of Akt, RASSF1A, and PTEN. B, HCC1937 cells were cotransfected with the indicated combinations of wild-type PTEN, phosphatase-dead PTEN C124S, and siRNAs for Raf-1 (*siRNA Raf-1*) or a control (*siRNA C*). MST2 immunoprecipitates were analyzed by Western blotting using antibodies as stated. MST2 kinase activity was tested by in-gel kinase assays. Cellular extracts (10 μ g) were analyzed by Western blotting using the indicated antibodies. C, Akt was downregulated by siRNA. Cell extracts, Raf-1, and MST2 immunoprecipitates were analyzed by Western blotting with the indicated antibodies. D, cells were cotransfected with Myc- and Flag-tagged MST2 and either wild-type PTEN (*wt*) or phosphatase-dead mutant PTEN (C124S). Immunoprecipitation and Western blot analysis were performed as described above.

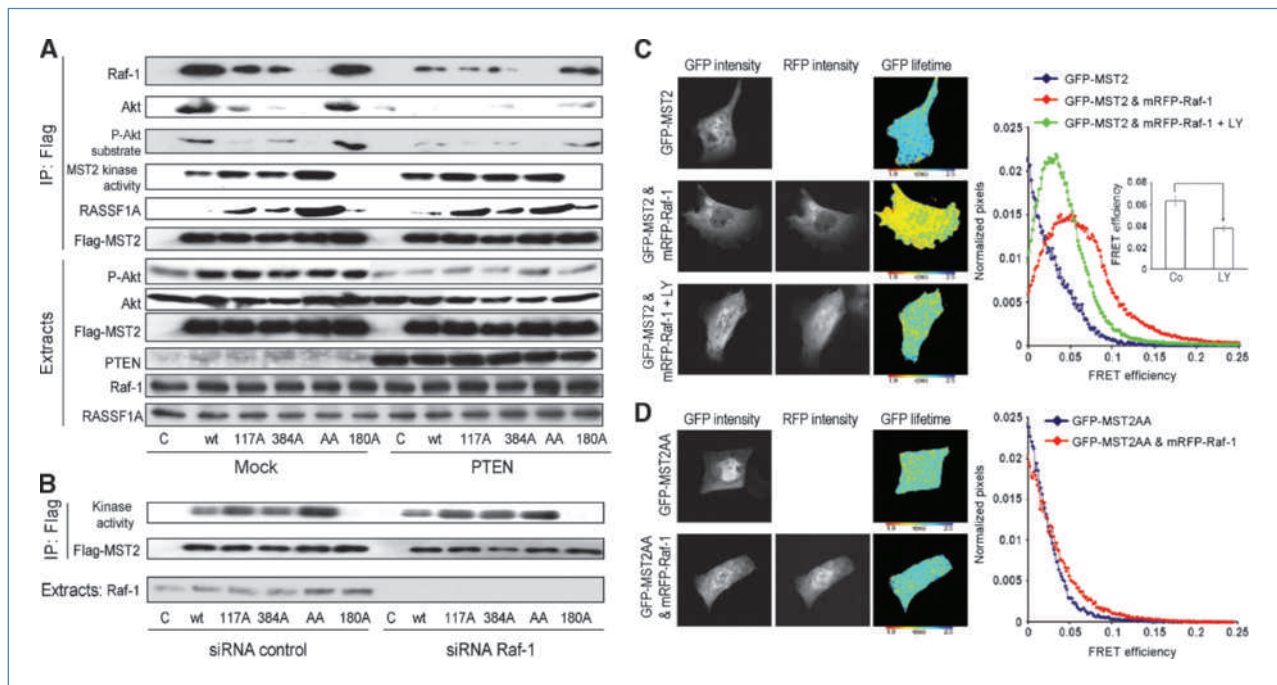


Figure 4. MST2 phosphorylation by Akt at T117 and T384 regulates MST2 kinase activity and Raf-1 interaction. **A**, HCC1937 cells were transfected with empty vector (C), Flag-tagged MST2 wild-type (*wt*), single mutants T117A and T384A (117A and 384A), the double mutant T117/384AA (AA), or the inactive mutant T180A (180A). PTEN was coexpressed where indicated. Flag immunoprecipitates were analyzed by Western blot using specific antibodies as stated. MST2 kinase activity was determined by in-gel kinase assays. **B**, cells were cotransfected with Flag-tagged MST2 mutants and control (*siRNA control*) or Raf-1-specific (*siRNA Raf-1*) siRNAs. MST2 immunoprecipitates were analyzed for kinase activity. **C**, cells were cotransfected with GFP-MST2 or the GFP-MST2 double mutant T117/384AA and mRFP1-Raf-1. The interaction between MST2 and Raf-1 was imaged by FLIM in cells treated with or without LY294002 (5 μ mol/L, 2 h). FRET between GFP and mRFP1 results in shortening of the fluorescent lifetime (τ) of GFP. The graph shows FRET efficiency calculated using the control lifetime (GFP alone) and effect of LY294002 (11 cells per group; *inset*). Statistical analysis was performed using Student's *t* test. **D**, FLIM of cells coexpressing the GFP-MST2 double mutant T117/384AA and mRFP1-Raf-1.

phosphorylation site mutants. Wild-type MST2 caused a small but significant increase of apoptosis, which was further increased when the single or double MST2 mutants were expressed. The inactive MST2 mutant T180A was unable to trigger apoptosis (Fig. 6C). Consistent results were obtained using caspase activation to monitor apoptosis (Supplementary Fig. S5). Similarly, in the *PTEN*^{-/-} HCC1937 cells, overexpression of wild-type MST2 and the single mutants T117A and T384A stimulated apoptosis (Fig. 6D). Moreover, PTEN reexpression slightly enhanced apoptosis (Fig. 6D). As in HeLa cells, the MST2 double mutant displayed the strongest proapoptotic activity, and this effect was resistant to PTEN expression.

To test the effects of MST2 mutations on other downstream pathways, we examined the activation of JNK and p38 (Supplementary Fig. S6), as they have been implicated in proapoptotic MST signaling (27–29). Expression of the MST2 single mutants and especially the double mutant induced JNK and, to a lesser extent, p38 activation. These effects were enhanced by the reexpression of PTEN. The kinase-inactive MST2 T180A mutant did not stimulate p38 and JNK under any condition. These results further confirm that Akt is critical in inhibiting MST2 and downstream signaling into different pathways.

Discussion

Our results provide evidence that MST2 is a hub that integrates the output activities of three pathways (i.e., the Raf-1, RASSF1A, and Akt pathways). This conclusion is based on the following evidence.

We show that MST2 is a physiologic substrate of Akt. Endogenous MST2 is phosphorylated by Akt in both serum-stimulated and *PTEN*^{-/-} cells. In quiescent cells, we have previously described that Raf-1 interacts with MST2 to inhibit its proapoptotic activity (6, 12). Interestingly, we have previously also observed that mitogens can disrupt the MST2–Raf-1 interaction without leading to activation of MST2 (12). Our current results explain this conundrum, suggesting the following model. In quiescent cells, MST2 is inhibited by being sequestered in a complex with Raf-1. Proapoptotic stimuli, such as Fas activation, staurosporine, or RASSF1A, break up this complex and induce MST2 activation by promoting RASSF1A binding and homodimerization (6, 12). It is not entirely clear how proapoptotic stimuli dissociate the MST2–Raf-1 complex. The Raf-1 and RASSF1A interaction sites in MST2 overlap and these proteins compete for MST2 binding (6), suggesting that proapoptotic stimuli that suppress Akt activity and MST2

phosphorylation will shift MST2 affinity from Raf-1 to RASSF1A. Mitogen stimulation and oncogenic Ras also dissociate the MST2-Raf-1 complex; however, due to the simultaneous activation of Akt and Akt-mediated MST2 phosphorylation, MST2 remains inhibited. This latter mechanism is especially relevant in *PTEN*^{-/-} cells, which display high Akt activity and hence efficient MST2 inhibition. *PTEN* is one of the most frequently altered tumor suppressor genes in human cancer (19). As loss of *PTEN* expression or function will interfere with MST2 activation and proapoptotic activity, MST2 may have a role as effector of the *PTEN* tumor suppressor to prevent unlicensed cell proliferation in normal cells and tissues.

It seems that the basal level of Akt activity is sufficient to phosphorylate MST2 and is at least in part responsible for MST2 binding to Raf-1 under nonstimulated conditions. The interaction of endogenous MST2 with Raf-1 is diminished when the basal levels of active Akt in quiescent cells are reduced by reexpressing *PTEN*, pharmacologic inhibition of the PI3K-Akt pathway, specific downregulation of Akt expression, or mutation of the Akt phosphorylation sites. Interestingly, Akt-induced phosphorylation of MST2 is not dependent on Raf-1, suggesting that Akt phosphorylation of

MST2 takes place before Raf-1 binding and promotes Raf-1-MST2 complex formation.

Previous work with the related MST1 kinase showed that Akt phosphorylation occurs only at the COOH-terminal site (15). Based on mutational analysis, our study clearly shows that in MST2 both NH₂- and COOH-terminal Akt motifs are phosphorylated. Indeed, the single MST2 mutants remained competent for interaction with Raf-1 and Akt, albeit to a lower extent than wild-type MST2. These single mutants also were more active and showed stronger interaction with RASSF1A than wild-type MST2 but to a lower extent than the double mutant T117/384AA. The double mutant showed a higher kinase activity and was more proapoptotic than the single mutants. These data show that Akt phosphorylation of both NH₂- and COOH-terminal sites is necessary for full regulation of MST2 by Akt, whereas phosphorylation of the COOH-terminal site is sufficient for regulation of MST1. Importantly, the regulation of MST1 and MST2 by Akt differs fundamentally. Akt phosphorylation prevents MST1 activation by caspase cleavage and selectively inhibits MST1 kinase activity toward FOXO3 but not histone 2B (15). In contrast, as our data show, Akt inhibits MST2 by a dual mechanism (i.e., shifting its binding from RASSF1A to Raf-1) and by directly

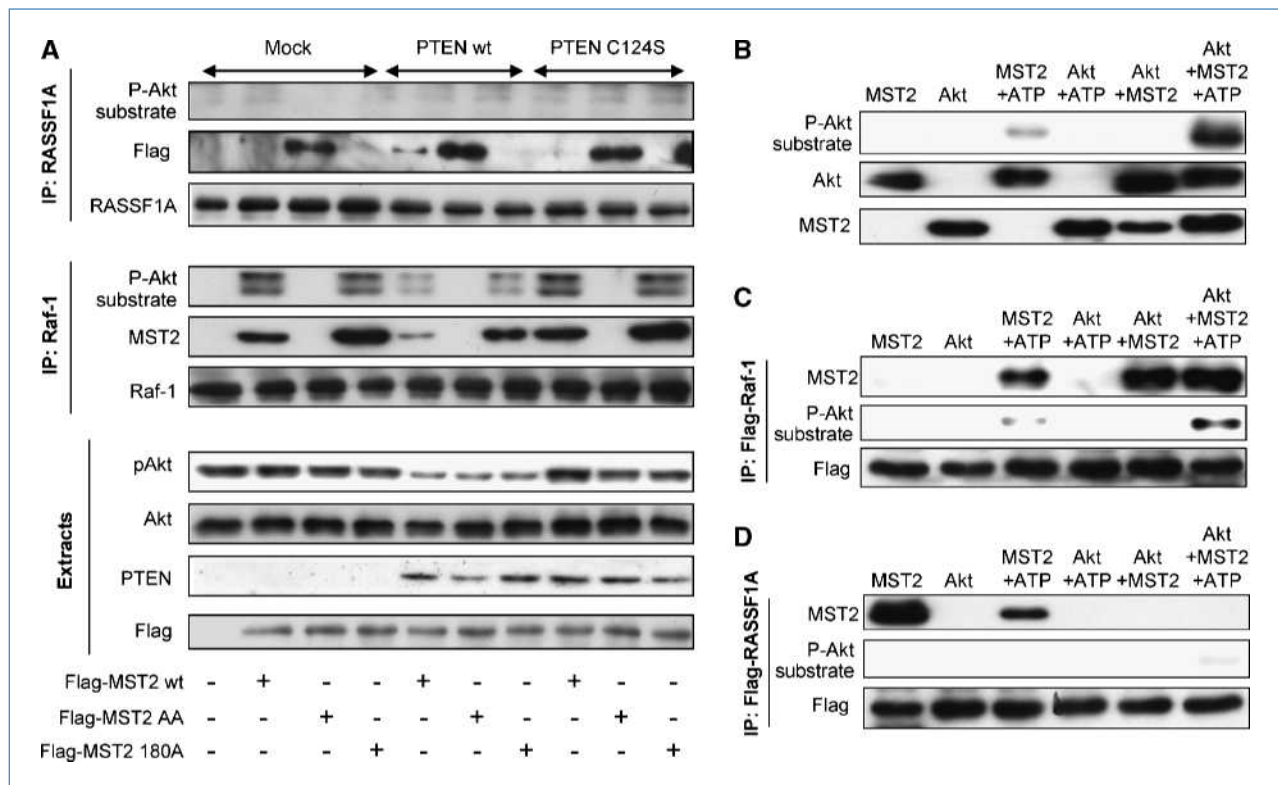


Figure 5. Akt phosphorylates Akt directly and prevents binding to RASSF1A. A, HCC1937 cells were transfected with empty vector, Flag-tagged wild-type MST2 (wt), the double mutant T117/384AA (AA), or the inactive mutant T180A (180A). *PTEN* wild-type or the phosphatase-dead mutant C124S were coexpressed as indicated. RASSF1A and Raf-1 immunoprecipitates and 10 μ g of cellular extracts were analyzed by Western blotting using the indicated antibodies. B, purified MST2 was incubated with purified Akt \pm ATP in a total volume of 100 μ L. Aliquots (2 μ L) were immunoblotted with the indicated antibodies. The remaining samples were divided into two and incubated with Flag-Raf-1 (C) or Flag-RASSF1A (D) immunoprecipitates. After washing MST2, proteins bound to the immunoprecipitates were examined with the indicated antibodies.

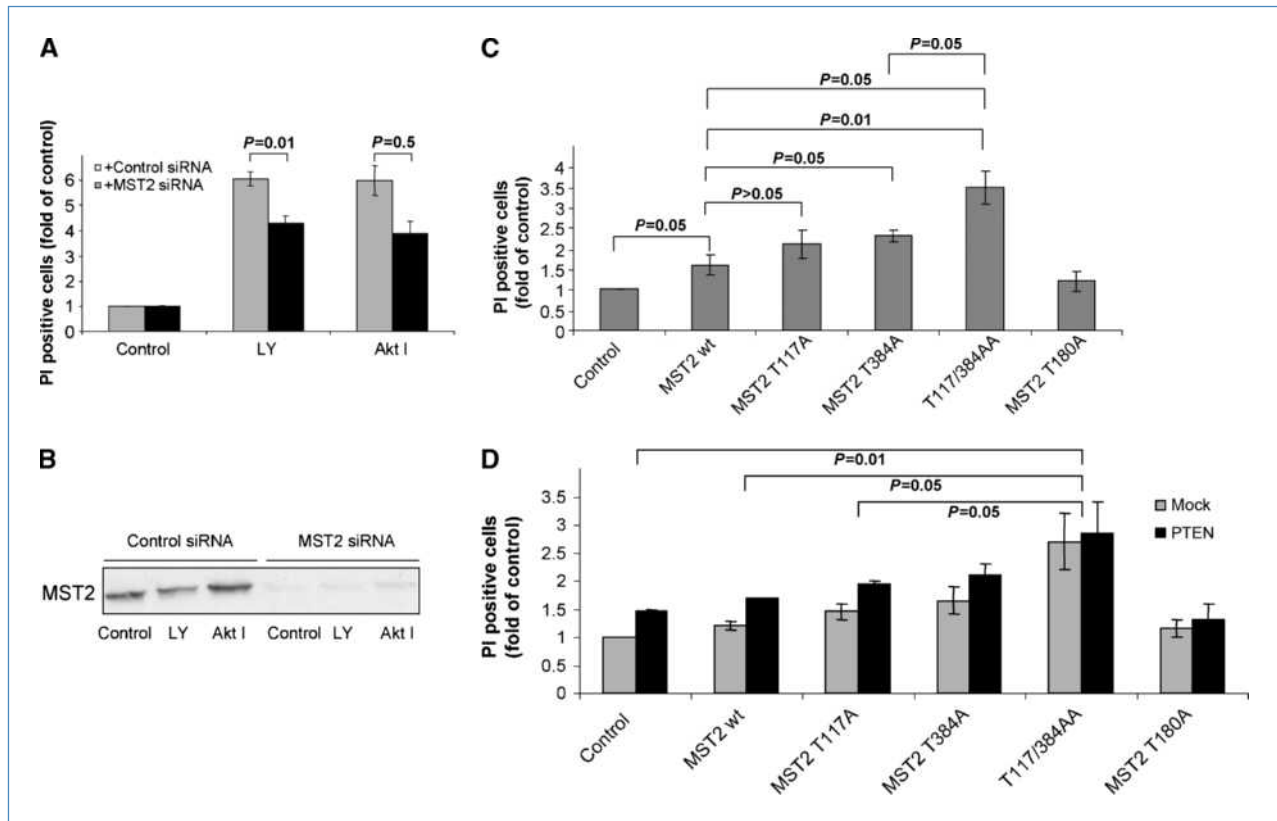


Figure 6. Akt protection against apoptosis involves MST2 inhibition. A, HeLa cells were transfected with control or MST2 siRNA and treated or not (Control) for 15 h with 5 μ mol/L LY294002 or 5 μ mol/L Akt inhibitor. Apoptosis was quantified by assaying DNA fragmentation as described in Materials and Methods. Statistical analysis was performed using Student's *t* test ($n = 4$). B, 10 μ g of cellular extracts were analyzed for full-length MST2 expression by Western blot. C, HeLa cells were transfected with empty vector (Control), Flag-tagged wild-type MST2 (wt), MST2 single mutants T117A and T384A (117A and 384A), the double mutant T117/384AA (AA), or the inactive mutant T180A (180A). Apoptosis was measured as above. Statistical analysis was performed using Student's *t* test ($n = 5$). There were no statistically significant differences between control and the T180A mutant, and wild-type MST2 and T180A. D, HCC1937 cells were cotransfected with MST2 constructs as described above with or without (Mock) PTEN. Apoptosis was measured as above.

inhibiting MST2 kinase activity independent of proteolytic cleavage. The relevant mechanism is inhibition of MST2 homodimerization, which is an early activation step that precedes activation by proteolytic cleavage.

Akt also has been reported to phosphorylate and inhibit YAP (18), a transcription factor that functions downstream of MST2 (30). Thus, Akt could regulate the pathway at several levels. Most importantly, our data suggest that MST2 is a point of coordination between the Raf, RASSF1A, and Akt pathways, with successful proliferation in response to Raf-1 stimulation requiring concomitant Akt activation. MST2 release is part of normal mitogenic regulation and maybe a safeguard against unlicensed proliferation by coupling mitogenic signaling with priming of an apoptotic signal. Intriguingly, Raf-1 and Akt can be differentially recruited by Ras protein isoforms. H-Ras is more effective in activating the PI3K-Akt pathway, whereas N-Ras and K-Ras are more potent in activating ERK (31). Therefore, on mitogen stimulation, MST2 release from Raf-1 should be triggered by Ras isoforms that preferentially bind Raf-1 and signal to ERK, whereas PI3K would be recruited by distinct Ras isoforms

to activate Akt. Such coordination would ensure that Raf-1 and Akt activation proceeds in parallel by avoiding competition between Raf-1 and PI3K for binding to the same Ras effector sites. In summary, these data suggest that MST2 is a point of convergence for Ras signaling that regulates the balance between proliferation and apoptosis by serving as a common regulation target for the Raf-1 and Akt pathways.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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