The Hippo pathway plays an important role in developmental biology, mediating organ size by controlling cell proliferation through the activity of a core kinase cassette. Multiple upstream events activate the pathway, but how each controls this core kinase cassette is not fully understood. Activation of the core kinase cassette begins with phosphorylation of the kinase MST1/2 (also known as STK3/4). Here, using a combination of in vitro biochemistry and cell-based assays, including chemically induced dimerization and single-molecule pulldown, we revealed that increasing the proximity of adjacent kinase domains, rather than formation of a specific protein assembly, is sufficient to trigger autophosphorylation. We validate this mechanism in cells and demonstrate that multiple events associated with the active pathway, including SARAH domain–mediated homodimerization, membrane recruitment, and complex formation with the effector protein SAV1, each increase the kinase domain proximity and autophosphorylation of MST2. Together, our results reveal that multiple and distinct upstream signals each utilize the same common molecular mechanism to stimulate MST2 autophosphorylation. This mechanism is likely conserved among MST2 homologs. Our work also highlights potential differences in Hippo signal propagation between each activating event owing to differences in the dynamics and regulation of each protein ensemble that triggers MST2 autophosphorylation and possible redundancy in activation.

The Hippo pathway regulates a variety of biological processes, ranging from control of organ size during development to decisions of cell fate and the suppression of tumorigenesis (1–5). In recent years, the Hippo pathway has emerged as a potential therapeutic target for the treatment of cancer and stimulation of tissue regeneration (6, 7). The Hippo pathway is a growth control pathway that is conserved from mammals to flies; the names of individual proteins, however, are different. Mammalian genes can rescue the phenotypes of flies lacking homologous genes (8–10). The pathway was originally identified in Drosophila as a pathway that, when disrupted, leads to overgrowth phenotypes (8, 11–16). The role of this pathway is conserved in mammals, as disruption of the pathway in mouse models leads to tumorigenesis (17–20).

In contrast to most canonical signaling pathways, the Hippo pathway is not activated by a single ligand/receptor pair. Instead, a variety of signals activate the pathway, including cell–cell contacts, the extracellular matrix, nutrients, stress, and G protein–coupled receptors (GPCRs), among others (5, 21). Despite the diversity of these inputs, each ultimately controls gene expression by regulating the cellular localization of the transcriptional co-factors YAP/TAZ (10, 18, 22–26). When the Hippo pathway is inactive, YAP/TAZ translocate to the nucleus and form a transcriptionally competent complex with TEAD. When the pathway is active, YAP/TAZ are phosphorylated, sequestered in the cytoplasm, and degraded. At the heart of the Hippo pathway is a core kinase cassette that includes two kinases that each have two isoforms, MST1/2 (also known as STK3/4 in mammals or Hippo in flies) and LATS1/2 (Warts in mammals, Mst1/Mst2 in flies) (27). Active MST1/2 phosphorylates and activates LATS1/2, and an activated MST1/2 phosphorylates and activates LATS1/2, and an active Lats1/2 phosphorylates YAP/TAZ (27–30).

Signaling through the core kinase cassette begins with activation of MST1/2, a member of the Sterile 20–like kinase family, a kinase that contains an N-terminal kinase domain followed by a disordered linker and coiled-coil domain termed SARAH (Fig. 1A). Active MST1/2 is phosphorylated on the activation loop (Thr-183 for MST1 and Thr-180 for MST2), in an event primarily attributed to trans-autophosphorylation (31–34) (Fig. 1A). Tao1 kinase can also phosphorylate the activation loop of both MST1 and MST2 but is likely not the primary route of MST1/2 activation (35, 36). Knockouts of Tao-1 in flies have a milder overgrowth phenotype than knockouts of hippo (35), and in mammalian cells Tao-1 knockouts do not block MST1/2 activation (37).

An unresolved question in the field is how multiple signals control the activity of the same core kinase cassette. Whereas multiple events that promote MST1/2 activation have been identified, a common molecular mechanism linking these events to autophosphorylation has not yet been identified. One line of evidence suggests that SARAH domain–mediated homodimerization is required for autophosphorylation (4, 31–34, 38–40). The characteristic overgrowth phenotype...
of flies with disrupted Hippo signaling can be recapitulated in flies bearing an allele of hippo lacking the SARAH domain (15). In cells, lower levels of activation loop phosphorylation are detected for both MST1 variants lacking the SARAH domain and Hippo variants bearing site-specific mutations that weaken SARAH domain homodimerization (38, 39). In solution, MST2 variants lacking a SARAH domain have little detectable autophosphorylation (31).

A set of membrane-associated proteins link external stimuli to the activation and membrane recruitment of the core kinase cassette. External stimuli signal through a set of membrane-associated proteins, NF2 (Merlin in flies) and Kibra, to activate the core kinase cassette (41–48). Overexpression of these upstream components stimulates pathway activity (42, 43, 49, 50), and knockdown of these proteins reduces Hippo homodimerization (32, 49). Both core kinases, MST1/2 and LATS1/2, are recruited to the membrane (48, 51), and their kinase activities are stimulated by engineering the membrane localization of the core kinase cassette to the membrane (32, 52, 53). In flies, whereas Hippo and Warts have a diffuse distribution in cells, phosphorylated Hippo is localized at the membrane (49, 50).

Whereas membrane recruitment of the core kinases may be a key step in signal propagation, the rationale for how changes in localization modulate signal transduction varies. Some attribute the increase in activity to co-localization of MST1/2 with its substrate, LATS1/2 (8, 13, 16, 48, 54, 55). Others suggest that membrane recruitment increases the local concentration of Hippo, which in turn promotes homodimerization and autophosphorylation (32, 39, 56). However, membrane recruitment of MST1/2 is incompatible with SARAH domain–mediated MST1/2 homodimerization, as membrane recruitment of MST1/2 relies on SARAH domain–mediated complex formation with SAV1 (8, 16, 48, 51, 54, 57–59).

The level of activated MST2 is elevated in the presence of its binding partner SAV1 (8, 16, 54, 57–59). SAV1 mediates membrane recruitment of MST1/2 (48), and this increases pathway activity (48). SAV1 can also simultaneously bind to and inhibit the activity of the phosphatase-containing complex STRIPAK, thereby contributing to MST1/2 activation by extending the t½ of phosphorylated MST2 (58). The MST1/2:SAV1 complex is mediated by a heterodimeric interaction between the SARAH domain of each protein SARAH domain, which begs the question of how MST1/2 becomes autophosphorylated in the presence of SAV1, as complex formation with SAV1 would block SARAH domain–mediated homodimerization of MST1/2 (58, 59). Adding to the confusion is the fact that Hippo SARAH domain homodimers are 4 orders of magnitude less stable than Hippo:Salvador SARAH domain heterodimers (60), suggesting that MST1/2 is unlikely to homodimerize in the presence of SAV1. In vitro MST2 autophosphorylation can be stimulated by a truncated variant of SAV1 that forms a 2:2 complex with MST2 (58). However, this fragment failed to increase autophosphorylation of MST2 in cells, which led to the conclusion that every domain of SAV1 is required for activation (58).

To understand how multiple events promote MST2 activation, we investigated the nature of MST2 autophosphorylation. We present a biochemical analysis of MST2 activity, which revealed a truncated variant of SAV1 that forms a 2:2 complex with MST2 (58). However, this fragment failed to increase autophosphorylation of MST2 in cells, which led to the conclusion that every domain of SAV1 is required for activation (58).
Results

**MST2 autophosphorylation requires only the kinase domain but is stimulated by the SARAH domain**

To understand how each domain of MST2 contributes to autophosphorylation of the activation loop, we asked which domains are required for this activity. We tested the ability of a set of MST2 variants corresponding to full-length MST2 (MST2-FL), the kinase and linker domains (MST2-KL), or the kinase domain (MST2-K) to undergo autophosphorylation. Each protein was purified in the unphosphorylated state and incubated with ATP and Mg^{2+}, and the level of phosphorylation at Thr-180 was monitored by Western blotting using direct detection of fluorescently labeled secondary antibodies. Each variant reached an equivalent level of autophosphorylation (Fig. 1B). These results demonstrate that the kinase domain alone is sufficient for autophosphorylation.

We next asked whether the linker and SARAH domains contributed to the rate of autophosphorylation. We repeated our *in vitro* autophosphorylation assay but monitored phosphorylation of each of the three MST2 variants (MST2-FL, MST2-KL, and MST2-K) as a function of time (Fig. 2). MST2-FL underwent autophosphorylation faster than either MST2-KL or MST2-K, and there was no significant difference between the rates of MST2-KL and MST2-K. Although not required for autophosphorylation, the SARAH domain stimulated MST2 activation, and the linker region did not make a significant contribution.

**Increasing kinase domain proximity stimulates MST2 autophosphorylation**

The higher rate of MST2-FL autophosphorylation is likely a consequence of SARAH domain–mediated homodimerization. We wanted to determine the molecular mechanism behind this stimulation that could be attributed to either specific contributions of the SARAH domain–mediated homodimerization or an increase in proximity of MST2 following homodimerization. To distinguish between allostery and proximity, we compared autophosphorylation of monomeric and dimeric MST2 variants lacking a SARAH domain. If activation is stimulated by an allosteric interaction involving the SARAH domains, then variants lacking a SARAH domain would have the same activity regardless of their oligomeric state. If activation is stimulated by proximity of the kinase domains, then dimerization of MST2 variants lacking a SARAH domain should result in increased autophosphorylation.

We employed a chemically induced homodimerization system in which we could switch the oligomeric state of MST2 from monomeric to dimeric forms and simultaneously monitor autophosphorylation. We expressed and purified a set of MST2 variants fused to a variant of the FK506-binding protein (FKBP_{36V}) that is monomeric in solution and homodimerizes in the presence of the rapamycin analog AP20187 (61). These variants corresponded to full-length (MST2-FL-FKBPF_{36V}), the kinase linker (MST2-KL-FKBPF_{36V}), or kinase domain (MST2-K-FKBPF_{36V}). We then compared the level of autophosphorylation for each of these fusion proteins in the presence or absence AP20187 and compared the activity with that of the equivalent MST2 variants lacking FKBPF_{36V} (MST2-FL, MST2-KL, MST2-K) (Fig. 3). In the absence of AP20187, the patterns of autophosphorylation for equivalent variants of MST2 either alone or fused to FKBPF_{36V} were the same, demonstrating that the basal activity of MST2 was not altered by fusion to FKBPF_{36V}. Additionally, the levels of autophosphorylation for MST2-FL and MST2-FL-FKBPF_{36V} were similar following the addition of either ATP or ATP and AP20187, indicating that the rapamycin analog, and subsequent dimerization, did not further stimulate autophosphorylation. Autophosphorylation, however, was stimulated following the addition of AP20187 and ATP for both fusion proteins lacking the SARAH domain, MST2-KL-FKBPF_{36V} and MST2-K-
FKBP<sup>F36V</sup>. Therefore, autophosphorylation of MST2 can be stimulated by dimerization in the absence of the SARAH domain, and SARAH domain–mediated allosteric interactions are not required to stimulate autophosphorylation.

**Homodimerization of MST2 in cells triggers autophosphorylation**

We wanted to determine whether the molecular mechanism determined in vitro is consistent with known events associated with the active Hippo pathway in cells. To investigate the molecular mechanism behind MST2 homodimerization in cells, we asked whether in cells the stoichiometry of MST2 variants influenced activation loop phosphorylation. HEK293 cells were transiently transfected with HA epitope–tagged MST2 variants corresponding to full-length MST2 (HA-MST2-FL) or truncated variants corresponding to the kinase linker (HA-MST2-KL) or isolated kinase domain (HA-MST2-K) as well as a MST2-FL protein bearing a kinase-inactivating substitution (HA-MST2-FLD146N). Cell lysates were analyzed by Western blotting to determine the relative fraction of MST2 phosphorylated on the activation loop (Thr-180) (Fig. 4A). More HA-MST2-FL was phosphorylated at pT180 than either variant of MST2 lacking a SARAH domain (HA-MST2-KL and HA-MST2-K). HA-MST2-FLD146N had minimal phosphorylation of Thr-180, which is attributed to the activity of the constitutive MST1/2 in the cells, suggesting that the activation loop phosphorylation detected was a consequence of autophosphorylation of the transfected MST2. To directly determine the stoichiometry of the variants determined by using single-molecule total internal reflection fluorescence microscopy (TIRF) and monitoring the number of photobleaching steps (Fig. 4B). We determined the stoichiometry of MST2 variants fused to mCherry corresponding to full-length (mCherry-MST2-FL), kinase linker (mCherry-MST2-KL), and kinase domain (mCherry-MST2-K) (Fig. 4, C and D). The majority of isolated mCherry-MST2-FL molecules underwent two photobleaching steps consistent with a dimeric assembly, whereas the majority of MST2 variants lacking the SARAH domain (mCherry-MST2-KL and mCherry-MST2-K) underwent a single photobleaching step, indicating a monomeric state.

**Recruitment of MST2 to a membrane triggers activation loop autophosphorylation**

As membrane localization of the core kinase cassette is associated with an active Hippo pathway (32, 52, 53), we wanted to determine how membrane recruitment of MST2 contributes to signal transduction. We hypothesized that membrane recruitment would increase the effective concentration of MST2 kinase domains and, in turn, trigger MST2 autophosphorylation and activation. First, we asked whether recruitment of MST2 to a lipid membrane was sufficient to stimulate autophosphorylation. Assembly of signaling complexes at the membrane can be recapitulated in vitro by binding His-tagged proteins to unilamellar vesicles containing lipids with nickel-chelated headgroups (64, 65). We monitored autophosphorylation of His-tagged MST2 variants corresponding to full-length MST2 (H6-MST2-FL), kinase linker (H6-MST2-KL), or the isolated kinase domain (H6-MST2-K) in the absence or presence of lipid vesicles made with either 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) or a mixture of DOPC and the nickel-chelated lipid 1,2-dioleoyl-sn-glycero-3-[N-(5-amino-1-carboxypentyl)iminodiacetic acid]succinyl] nickel salt (DGS-NTA(Ni))
The level of autophosphorylation for each MST2 variant following incubation with ATP did not change upon the addition of the DOPC vesicles. In contrast, autophosphorylation increased following the addition of vesicles with nickel-chelated headgroups for MST2 variants lacking the SARAH domain (H6-MST2-KL and H6-MST2-K). These data suggest that simply increasing the effective local concentration of MST2, here achieved through recruitment to the surface of a vesicle, stimulates autophosphorylation.

We then asked whether an MST2 autophosphorylation could be stimulated by recruitment to the plasma membrane. Membrane recruitment of proteins can be controlled using an inducible translocation system in which cells are transiently transfected with a membrane-targeted variant of the rapamycin-binding domain of mTOR (Lyn11-FRB) and the protein of interest fused to FKBP (66–68) (Fig. 6A). The addition of a membrane-permeable rapamycin analog, AP21967, promotes binding of FKBP to FRB and causes the membrane translocation of the FKBP fusion protein. We transiently transfected HEK293 cells with plasmids encoding MST2 variants corresponding to full-length MST2 (HA-MST2-FL) or kinase linker (HA-MST2-KL) alone or fused to FKBP (HA-MST2-FL-FKBP and HA-MST2-KL-FKBP) and Lyn11-FRB as indicated (Fig. 6B). We first confirmed that the pattern of activation loop phosphorylation of MST2 was not altered either by co-expression with Lyn11-FRB, by fusion of MST2 to FKBP, or by the addition of AP21967 in the absence of Lyn11-FRB (Figs. 4A and 6B). We then analyzed whether inducing MST2-FKBP fusions
to the membrane following the addition of AP21967 altered autophosphorylation. The addition of AP21967 resulted in a 2.5-fold increase in the fraction of phosphorylated MST2-KL-FKBP but no change for MST2-FL-FKBP. We then confirmed the cellular localization of both Lyn11-FRB and HA- and FKBP-tagged MST2 variants in the presence and absence of both ATP and vesicles containing lipids with or without a nickel-chelated headgroup, as indicated. The level of autophosphorylation was monitored by Western blotting, and total protein was measured by Coomassie-stained SDS-PAGE. The experiment was performed three times, and a representative set is shown. Migration of molecular mass markers, in kDa, is indicated to the left of each gel. Bottom, the relative, normalized fraction of phosphorylated MST2 is plotted as black circles below the corresponding lane, and a bar graph shows the mean value with error bars indicating S.D. from three independent experiments. Significant differences are calculated using an unpaired t test (***, p ≤ 0.001; ns, p ≥ 0.05).

Figure 5. Recruitment of MST2 to the surface of vesicles triggers autophosphorylation. A, cartoon illustrating the differences in the effective concentration of H6-MST2-K (white) either in solution (left), mixed with DOPC (gray) vesicles (middle), or mixed with vesicles with nickel-chelated headgroups (green) (right). B, top, purified H6-MST2-FL (blue), H6-MST2-KL (light blue), and H6-MST2-K (white) were incubated in the presence or absence of both ATP and vesicles containing lipids with or without a nickel-chelated headgroup, as indicated. The level of autophosphorylation was monitored by Western blotting, and total protein was measured by Coomassie-stained SDS-PAGE. The experiment was performed three times, and a representative set is shown. Migration of molecular mass markers, in kDa, is indicated to the left of each gel. Bottom, the relative, normalized fraction of phosphorylated MST2 is plotted as black circles below the corresponding lane, and a bar graph shows the mean value with error bars indicating S.D. from three independent experiments. Significant differences are calculated using an unpaired t test (***, p ≤ 0.001; ns, p ≥ 0.05).

Figure 6. Membrane recruitment stimulates MST2 phosphorylation. A, cartoon of the chemically induced membrane translocation assay employed. B, HEK293T cells were transiently transfected with plasmids encoding HA-tagged MST2 variants and FLAG-tagged Lyn11-FRB, as indicated. For each condition, cells were treated either with carrier or AP21967. Top, normalized cell lysates were analyzed by Western blots to detect phosphorylated MST2 (pT180), the epitope tags (HA or FLAG) for expression, or β-actin as a loading control. The experiment was performed six times, and a representative set of blots is shown. Migration of molecular weight markers, in kDa, are indicated to the left of each gel. Bottom, band intensities were quantified, and the -fold change in the relative fraction of phosphorylated of MST2 was calculated ((pT180/HA)1 AP21967/(pT180/HA)2 AP21967). Individual data (black circles) are plotted below each lane, and the bar graph represents mean values with error bars corresponding to the S.D. from the six biological replicates with data from cells treated with AP21967 in orange and with mock in white. Significant differences were calculated using a paired t test (**, p ≤ 0.01; ns, p ≥ 0.05). C, HEK293 cells were transfected with the indicated plasmids, treated either with mock or AP21967 as indicated, and stained with antibodies that recognized either the FLAG (red) or HA (green) epitope tags or with DAPI (blue). Representative images are shown. Scale bars, 10 μm.
EDITORS’ PICK: Increasing proximity triggers MST2 autophosphorylation

**Higher-order complex formation with SAV1 stimulates MST2 autophosphorylation**

We wondered what triggered MST2 autophosphorylation in the presence of SAV1 and hypothesized that autophosphorylation could be stimulated by an increase in concentration following the formation of a higher-order complex with SAV1. To determine the stoichiometry of the complex between full-length SAV1 and MST2-FL in cells, we again used SiMPull. HEK293 cells were transiently transfected with plasmids encoding SAV1-FL fused to monomeric yellow fluorescent protein (mYFP-hSav1-FL) and MST2-FL fused to mCherry (mCherry-MST2-FL). Complexes were isolated by immunoprecipitation using antibodies that recognize either mYFP (Fig. 7A) or mCherry (Fig. 7B), and the fluorescent intensity for both mYFP and mCherry was monitored over time. In cells, SAV1-FL and MST2-FL form a complex in which the majority of molecules have a 2:2 stoichiometry, independent of the order of pulldown. To determine whether higher-order complex formation with SAV1 promotes MST2 autophosphorylation *in vitro*, we purified H6-MST2-FL alone or in complex with SAV1 variants corresponding to either just the SARAH domain (SAV1-SARAH) or both WW domains and SARAH (SAV1-WW-SARAH). SAV1 contains an atypical WW domain capable of homodimerization (57, 69). We estimated the molecular mass of each complex by size-exclusion chromatography (Table 1 and Fig. 7C). Based on elution volumes, H6-MST2-FL alone forms a homodimer: when bound to SAV1-WW-SARAH a 2:2 complex and when bound to SAV1-SARAH a 1:1 complex. We then monitored autophosphorylation of H6-MST2-FL alone and in the presence of the different SAV1 variants (Fig. 7D). Autophosphorylation of HA-MST2-FL was higher for MST2 in higher-order complexes, both homodimers and 2:2 complexes with SAV1-WW-SARAH, than when in a 1:1 complex with SAV1-SARAH, in agreement with previous reports (58). These data suggest that SAV1 can trigger autophosphorylation of MST2 by bringing adjacent kinase domains into proximity following higher-order complex formation.

**Discussion**

We propose a molecular mechanism for MST2 autophosphorylation that is regulated by the effective local concentration of the kinase domains. Based on the sequence and functional similarities between MST1, MST2, and Hippo, we propose this mechanism to be conserved among these homologues. We demonstrate that *in vitro* autophosphorylation does not require the linker or SARAH domains (Fig. 1) but is stimulated by the SARAH domain (Fig. 2). We asked whether allosteric interactions between the kinase and SARAH domains or an increase in effective concentration following SARAH domain-mediated homodimerization was the source of this stimulation. We demonstrate that dimerization of MST2 variants lacking the SARAH domain replicates the increase in autophosphorylation observed between full-length MST2 and MST2 variants lacking the SARAH domain (Fig. 3). A molecular mechanism controlled by effective concentration is consistent with the proposed trans-autophosphorylation of MST2 (31–34). We then demonstrate that this molecular mechanism is consistent with cellular events associated with the activated Hippo pathway: MST2 homodimerization (Fig. 4), membrane recruitment (Figs. 5 and 6), and binding to SAV1 (Fig. 7).

In cells, tethering two MST2 kinase domains together via SARAH domain-mediated homodimerization promotes MST2 autophosphorylation. We show that in cells, the majority of transiently transfected MST2-FL homodimerizes and is phosphorylated, whereas variants lacking the SARAH domain were primarily monomeric and unphosphorylated (Fig. 4). This phosphorylation trend is recapitulated *in vitro* with purified proteins (Fig. 18). A kinase dimer that mediates trans-autophosphorylation has been proposed, yet attempts to identify residues required for both kinase domain dimerization and autophosphorylation have been unsuccessful, perhaps owing to the weak affinity ($K_d = 36 \mu M$) between MST2 kinase domains (31, 32, 39, 70). The molecular mechanism proposed here does not require a specific kinase dimer but is not in conflict with the formation of one. In fact, increasing proximity would further stabilize a weak dimer through an avidity effect resulting from increased effective local concentration and additional spatial constraints (71).

Membrane recruitment of both MST1/2 and the core kinase cassette propagates Hippo signaling. We demonstrate *in vitro* and in cells that membrane recruitment triggers autophosphorylation of MST2 (Figs. 5 and 6). Our results demonstrate that the increase in autophosphorylation can be attributed to the increased proximity of kinase domains rather than formation of a specific SARAH domain–mediated interaction. Clustering of signaling molecules at the membrane is a common mechanism for signal transduction and is estimated to increase the concentration of the protein by 3 orders of magnitude (72–74). Our vesicle experiments recapitulate a similar-magnitude increase in effective concentration of MST2. Whereas the assays included 0.5 μM bulk MST2 kinase domain, the calculated effective concentration of kinase domains on the surface of the vesicles is ~0.3 mM (65) (Fig. 5), and this thousand-fold increase in effective concentration induced autophosphorylation. Multiple models propose that membrane recruitment is activating, not as a consequence of catalytic changes, but by co-localizing MST1/2 with downstream targets (8, 13, 16, 48, 54, 55). Co-localization of a kinase with its substrate is a common occurrence in signaling pathways, as it provides both spatial regulation and additional specificity for the kinase (75). Whereas co-localization may contribute to signal transduction, our results demonstrate that membrane recruitment of MST1/2 has a catalytic contribution to signal propagation.

Based on the stabilities of SARAH domain–mediated complexes from *Drosophila*, MST1/2 is unlikely to form homodimers in the presence of SAV1 (60), so we asked whether autophosphorylation of MST2 could occur in the presence of SAV1 as a result of higher-order complex formation. In cells, it is difficult to deconvolute the specific contribution from any one region of SAV1 from the observed overall increase in MST1/2 autophosphorylation (48, 58). We used SiMPull to show that in cells full-length SAV1 and MST2 form a 2:2 complex (Fig. 7, A and B). Although we cannot, at present, fully rule out perturbations of the complex caused by either tagging or overexpression in our SiMPull analysis, our conclusions broadly agree with...
what previously was known about these proteins (13, 57, 58, 69, 76). Then we show that in vitro only SAV1 variants that form higher-order complexes of MST2-FL stimulate autophosphorylation (Fig. 7, B and C). In cells, SAV1 variants lacking either the atypical WW domain, which mediates SAV1 homodimerization, or the SARAH domain, which mediates complex formation with MST2, do not fully activate MST2 (58). Additionally, loss of SAV1 dimerization in cells also inhibits downstream...
signaling, as monitored by levels of LATS1/2 phosphorylation (76). This reduction in signal propagation is likely a consequence of having lower levels of activated MST2 as a direct consequence of blocking higher-order complex between SAV1 and MST2. Formation of a higher-order complex with SAV1 is sufficient to trigger MST2 autophosphorylation.

Activation of MST1/2 as a consequence of increased proximity will influence the dynamics and regulation of signal transduction. Our work suggests that multiple events could simultaneously promote MST1/2 autophosphorylation; for example, autophosphorylation could be triggered by both complex formation with SAV1 and membrane recruitment. This potential redundancy in activation would ensure a sustained, rather than transient, response to activating signals as well as provide a route for the pathway to be reactivated in the response to negative signals (77). The physical differences between events that increase proximity of MST1/2 kinase domains will affect both the level and lifetime of autophosphorylated MST1/2 and, thus, modulate the downstream response. Trans-autophosphorylation reactions inherently represent a positive feedback switch with the rate of activation dependent on the magnitude of the change in effective concentration (78–81). Not all activating events will result in the same change in effective concentration; for example, based on linker lengths, homodimerization of MST1/2 will raise effective concentration of MST1/2 higher than complex formation with SAV1 (82). Back-of-the-envelope calculations, using available structural data and assuming that disordered regions are fully extended peptide chains, reveal that the proximity of adjacent MST2 kinase domains in either an MST2 homodimer, a complex with SAV1, or an FKBP-mediated homodimer are all roughly within 1000 Å. Recent measurements of the relationship between linker length and effective concentrations suggest, however, that the relationship may not be linear (83). Precise measurements of the effective concentration of MST2 in cells for each signaling event will be required to fully understand the differences among the scenarios.

Because multiple MST2-containing complexes can trigger autophosphorylation rather than one specific protein complex, each of these assemblies will interact with a different subset of proteins. Each of these additional protein:protein interactions represent a potential node to regulate MST1/2 activity. Having an ensemble of events that activate MST2 to potentially different levels is similar to the partial or biased agonism observed for the responses of both Epidermal Growth Factor Receptor and GPCRs to an ensemble of ligands (84–88). These additional factors may explain reports of MST1/MST2 heterodimers having lower kinase activity that their respective homodimers (89).

Our work addresses how upstream signals activate the core kinase cassette of the Hippo pathway. We suggest a unifying molecular mechanism in which increasing the effective local concentration of MST1/2 kinase domains triggers autophosphorylation, rather than a specific protein assembly. Our findings reveal that multiple events associated with the active Hippo pathway increase the proximity of MST2 kinase domains and stimulate autophosphorylation. Re-evaluating cellular events associated with active Hippo signal transduction in light of this molecular mechanism reveals potential differences in both the response rate and duration of MST1/2 autophosphorylation as well as suggesting additional points of regulation via interactions with effector proteins. Our model also predicts that events that lower the effective concentration of MST1/2, such as a heterodimerization with RASSFs (34, 90) or delocalization of Scribble during the epithelial-to-mesenchymal transition (91), could also prevent autophosphorylation. Our model further predicts that events that increases proximity will promote autophosphorylation, and, beyond those events already discussed here, could also include the formation of MST1/2 condensates, as has been observed for other components of the kinase cassette (92). This model also reveals a potential route to develop tools that activate the pathway by increasing MST2 concentration, as demonstrated with chemically induced dimerization experiments (Figs. 3 and 6). Going forward, this framework will aid in comparison of activating events and explaining how pathway activity varies in response to different stimuli or environments.

### Experimental procedures

#### Protein expression and purification

DNA sequences corresponding to human MST2-FL (residues 1–484), MST2-KL (residues 1–433), or MST2-K (residues 1–314) were cloned into modified pBAD4 plasmids encoding either an N-terminal H6 and SUMO tag (93) or a C-terminal FKBP tag bearing an F36V substitution (FKBPF36V) (61) as well as into a pET-1B vector encoding an N-terminal H6 tag followed by a TEV cleavage site, which was a gift from Scott Gra-dia (Addgene plasmid 29653). T7 express cells were transformed with a pRSF-Duet plasmid (Millipore–Sigma) encoding an MBP-tagged α-phosphatase as well as either an MST2 variant in a pBAD4 vector or an MST2 variant in a pET vector and prARE (Millipore–Sigma). Cells were grown at 37 °C, and protein expression was induced with 0.5 mM isopropyl-β-D-1-thio-galactopyranoside (IPTG) when the cells reached OD600 of 0.8. Cells were further grown at 20 °C overnight. Cells were lysed in 50 mM Tris, pH 8.0, 400 mM NaCl supplemented with protease inhibitors (Sigma–Aldrich). Clarified lysate was incubated with nickel-charged Profinity–IMAC resin (Bio-Rad) for 1 h at 4 °C, and protein was eluted with 125 mM imidazole. Purified proteins bearing a SUMO tag were incubated with SENP protease to remove tags. Cleaved proteins as well as proteins bearing just a H6 tag were then further purified by anion exchange. If the protein was to be phosphorylated, MST2 variants were incubated with 5 mM ATP and 10 mM MgCl₂ for 30 min at room temperature, and the reaction was quenched by the addition of 20 mM EDTA. Both unphosphorylated and phosphorylated
MST2 were further purified by size-exclusion chromatography in 10 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM tris(2-carboxyethyl)phosphine (TCEP). Protein was concentrated to ~10 mg/ml and flash-frozen in liquid nitrogen. Expression and purification of unphosphorylated variants of MST2-FKBP<sup>F360</sup>V variants followed the same purification as above.

Complexes of MST2 and SAV1 were co-expressed and purified using tandem affinity purification. Nucleotides corresponding to either H6-TEV-MST2-FL or MBP-λ-phosphatase were cloned into pRSF-Duet. Nucleotides corresponding to either SAV1 WW-SARAH domain (residues 199–383) or SARAH domain (residues 269–383) were cloned into a modified pGEX-2TK (Sigma–Aldrich) downstream from an N-terminal GST tag followed by a PreScission protease (homemade) cleavage site. Rosetta2 (Millipore–Sigma) or T7 Express cells were transformed with plasmids containing MST2-FL-λ-phosphatase, and either SAV1 WW-SARAH or SARAH domain, respectively. Cells were grown to an OD<sub>600</sub> of 0.8, at which time protein expression was induced by the addition of the 0.25 mM IPTG and continued overnight at 16 °C. Cells were lysed in 40 mM Tris, pH 7.0, 400 mM NaCl, 5% glycerol, and 1 mM DTT. MST2-FL:SAV1 complexes were then isolated by sequential GSH affinity (Sigma–Aldrich) and immobilized nickel chromatographies. On-column cleavage of GST tag was completed by PreScission protease for 2 h at 4 °C. Complexes were further purified by size-exclusion chromatography in a final buffer of 20 mM Tris, pH 8.0, 200 mM NaCl, 5% glycerol, and 1 mM TCEP and stored flash-frozen in liquid nitrogen at ~2 mg/ml.

**In vitro autophosphorylation assays**

For single-time point measurements, 10 μM concentrations of the indicated unphosphorylated MST2 variants were incubated in 5 mM MgCl<sub>2</sub>, 0.5 mM MnCl<sub>2</sub>, 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 4% glycerol, 1 mM TCEP, and 10 mM Tris, pH 8.0, in the presence or absence of 5 mM ATP at room temperature for 30 min. Reactions were quenched with 20 mM EDTA, and samples were analyzed by Coomassie-stained SDS-PAGE and Western blotting using a primary antibody that recognizes MST2 phosphorylated at Thr-180 (Cell Signaling Technology, lot 1) and a secondary IRDye 800CW goat anti-rabbit antibody (LI-COR, lot C90220-06). Reactions were performed three times. The relative fraction phosphorylated was determined by dividing the intensity of the pT180 band by the intensity of the Coomassie band. The signal between blots was normalized to a loading control that was included on each blot. This assay represents the base protocol that was then modified for the following in vitro autophosphorylation experiments.

For time courses monitoring autophosphorylation, the autophosphorylation reaction included the following modifications. Reactions were carried out in 60 μl at room temperature and contained a 0.5 μM concentration of each indicated MST2 variant and 1 mM ATP in the same buffer as above. Samples were taken at 0, 1, 2.5, 5, 10, 30, 45, and 60 min and quenched with 20 mM EDTA. Reactions were performed three times.

For chemically induced homodimerization experiments, the autophosphorylation assay included the following modifications. 0.5 μM concentrations of the indicated unphosphorylated MST2 variants were incubated in either 0.2% ethanol or 0.2% ethanol supplemented with 2 μM AP20187 (TaKara Bio USA) at room temperature for 10 min and then quenched. Reactions were performed three times.

For autophosphorylation reactions in the presence of lipid vesicles, unilamellar vesicles were prepared and used on the same day in a single-time point autophosphorylation reaction with the following modifications. Reactions contained 0.5 μM of unphosphorylated H6-tagged MST2 and 0.4 mg/ml of lipid vesicles and were incubated on ice for 5 min. Reactions were initiated with the addition of 1 mM ATP, incubated at room temperature for 10 min, and quenched with 20 mM EDTA. These reactions were repeated three times. Estimations for the effective concentration of MST2 kinase domains on the surface of vesicles followed the method outlined previously (65).

**Image quantification and statistical analysis**

Both Western blots and Coomassie-stained gels were scanned on an Odyssey IR Imaging System (LI-COR), and band intensity was quantified by ImageJ (94). Prism (GraphPad software, La Jolla, CA) was used to plot data and for statistical analysis. A loading control was included on each blot when signal was normalized between blots.

**Preparation of unilamellar vesicles**

Small unilamellar vesicles were prepared as described previously (95). Briefly, DOPC (Avanti Polar Lipids) and DGS-NTA (Ni) (Avanti Polar Lipids) were dissolved in chloroform and dried under nitrogen. Lipids were rehydrated in 400 μl of 10 mM Tris, pH 8.0, 150 mM NaCl and briefly sonicated every 10 min for a total of 30 min in a 37–40 °C water bath. Vesicles were subsequently formed at 37–40 °C by extrusion of 400 μl of resuspended lipids through a 100-nm polycarbonate membrane (Avanti Polar Lipids) using an Avanti minietruder according to the manufacturer’s directions. The amount of total lipids used to make vesicles was either 0.40 mM DOPC or a mixture of 0.38 mM DOPC and 0.02 mM DGS-NTA(Ni) lipids.

**Cell culture and transfection**

HEK293T cells (ATCC, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 5% fetal bovine serum (VWR) and 2 mM glutamine at 37 °C and 5% CO<sub>2</sub>. Cells were transfected with the indicated plasmids using a 3:1 ratio of polyethylenimine “MAX” (Polysciences, Inc.) to DNA (96).

**Membrane recruitment in cells**

HEK239T cells were plated at 0.2 × 10<sup>6</sup> cells/ml and transfected with the indicated plasmids. 48 h following transfection, cells were treated either with 1 μM AP21967 dissolved in 0.2% ethanol or with an equivalent volume of 0.2% ethanol, as indicated, and incubated at 37 °C for 20 min. Following treatment, cells were lysed in ice-cold radioimmune precipitation buffer supplemented with 5 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM sodium pyrophosphate, 1 mM...
β-glycerophosphate, and Universal Nuclease (Thermo Fisher Scientific). Normalized cell lysates were analyzed by Western blotting using the following combinations of primary and secondary antibodies: α-phospho-MST1 (Thr-183)/MST2 (Thr-180) rabbit mAb (Cell Signaling Technologies, lot 1), IRDye 800CW goat α-rabbit (LI-COR, lot C90220-06), α-HA (Roche (Basel, Switzerland), lot 34502100), IRDye 680RD goat anti-rat (LI-COR, lot C71115-11), α-FLAG (Sigma–Aldrich, lot SLCD3990), IRDy 680RD goat anti-mouse (LI-COR, lot C81106-01), and α-β-actin (Abcam (Cambridge, UK), lot GR240004-1), and IRDye 800CW goat α-rabbit (LI-COR, lot C90220-06). The experiment was performed six times.

**Immunofluorescence staining**

HEK293T cells were plated at 0.1 × 10^6 cells/ml on polylysine-treated coverslips and 24 h later transiently transfected with the indicated plasmids. 48 h following transfection, cells were fixed with ice-cold 4% formaldehyde for 10 min, followed by permeabilization with PBS supplemented with 0.2% Triton X-100 for 10 min at room temperature. Cells were blocked with 2% BSA for 1 h at room temperature and then stained with either α-HA (Santa Cruz Biotechnology, Inc. (Dallas, TX), lot L0805) or α-FLAG (Sigma–Aldrich, lot SLCD3990) antibodies for 1 h at room temperature, followed by either Alexa Fluor 488 goat α-rabbit (Thermo Fisher Scientific, lot 2110498) or Alexa Fluor 594 goat α-mouse (Thermo Fisher Scientific, lot T1271722), respectively. Slides were stained by VECTASHIELD (Vector Laboratories) mounting reagent with 4′,6-diamidino-2-phenylindole (Vector Laboratories) and sealed with nail polish. A Zeiss Observer Z1 fluorescence microscope with a Zeiss Plan-Apochromat ×63 objective (numerical aperture 1.40) and an Apotome VH optical sectioning grid (Carl Zeiss, Jena, Germany) was used. Images were taken by a Zeiss AxioCam MRm camera and processed using AxioVision software.

**Gel filtration analysis**

200 μl of 25 μM MST2-FL, MST2-FL and SAV1 SARAH, or MST2-FL and SAV1 WW-SARAH were injected onto Superdex 200 Increase 10/300 (GE Healthcare) equilibrated in 20 mM Tris, pH 8, 200 mM NaCl, 5% glycerol, and 1 mM TCEP. Additionally, gel filtration standards (Bio-Rad) were run on the column in the same buffer. The partition coefficient (Kav) for each protein standard was calculated using the elution volume of each protein (Ve), the void volume of the column (Vo), and the total bed volume (Vt).

\[ K_{av} = \frac{(V_e - V_o)}{(V_t - V_o)} \quad (\text{Eq. 1}) \]

Kav was then plotted as a function of the log of the molecular weight. This calibration curves were then fit to a straight line. For each MST2 complex, Kav was calculated using its Ve and a molecular weight calculated using the calibration curve.

**Single-molecule imaging and spot counting**

Single-molecule experiments were performed on a prism-type TIRF microscope equipped with an electron-multiplying CCD camera (97). For single-molecule pulldown experiments, quartz slides and glass coverslips were passivated with 5000 molecular weight methoxy poly(ethylene glycol) (Laysan Bio Inc.) doped with 2–5% 5000 molecular weight biotinylated PEG (Laysan Bio Inc.). Biotinylated antibodies against YFP (Rockland Immunochemicals) or RFP (Abcam) were previously immobilized on the surface of the quartz slide via neutravidin-biotin linkage as reported previously (62). Each passivated slide and coverslip were assembled into flow chambers. Following transfection, cells were harvested and lysed in lysis buffer (20 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol) supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 5 mM NaF, 2.5 mM sodium pyrophosphate, 0.1 mM β-glycerophosphate and supplemented with Protease Inhibitor mixture (Sigma) and Universal Nuclease (Thermo Fisher Scientific). Clarified cell lysates were diluted to ~0.0005 mg/ml in lysis buffer supplemented with 0.2 mg/ml BSA. Fluorescently tagged proteins were isolated via pulldown with the appropriate biotinylated antibodies and washed twice in 50 μl of lysis buffer. The mCherry and mYFP fluorescent proteins were excited at 488 and 568 nm, respectively, and the emitted fluorescence signal was collected via band pass filters BL 607/36 (Semrock, NY) for mCherry and HQ 535/30 (Chroma Technology) for YFP. 15 frames were recorded from 20 different imaging areas (5000 μm²), and isolated single-molecule peaks were identified by fitting a Gaussian profile to the average intensity from the first 10 frames. Mean spot count per image for each signal was obtained by averaging 20 imaging areas using MATLAB scripts. All experiments were carried out at room temperature (22–25 °C).

**Single-molecule co-localization analysis**

Co-localization data were acquired from two separate movies from the same region of a slide using mYFP and mCherry. The co-localization criterion was set to a diffraction-limited region of ~300 nm, which corresponds to 3 pixels for this TIRF setup. The percentage of co-localization was calculated as the percentage of molecules that co-aligned with the pulled-down component. mYFP and mCherry images taken from different areas were also overlapped to determine the probability of false co-localization arising from random spatial overlap of single molecules. In a typical experiment, the surface density was maintained at ~600 molecules/5000-μm² imaging area for the pulled down component to reduce chances of false co-localization.

**Photobleaching analysis**

A single photobleaching step was characterized by an abrupt drop in fluorescence intensity. Single-molecule fluorescent time traces from individual YFP or mCherry spots were manually scored for the number of photobleaching steps, and the stoichiometry of the molecules was assigned based on the numbers of steps observed (62, 63). All images were collected at a time resolution of 100 ms. Each molecule was either arrayed based on the number of photobleaching steps (typically 1–4) or discarded if no distinct photobleaching step was identified. The stoichiometry of MST2 or SAV1:MST2 complexes was
determined by photobleaching the mCherry fluorophore then sequentially photobleaching the YFP fluorophore, and counting the photobleaching steps thereof. All spots with no fluorescent signal from either of the fluorophores were rejected. A minimum of 500 molecules acquired from at least three different imaging areas were analyzed for each experimental condition.

Data availability

All processed data are included in this article. Requests for raw data, further information, or reagents contained within the article are available upon request from the corresponding author, Jennifer Kavran (jkavran@jhu.edu).

Acknowledgments—We thank Dan Raben and Qianqian Ma for expertise and use of equipment to make uninamellar vesicles and Mike Matunis and Danielle Bouchard for help with immunofluorescence staining.

Author contributions—T. T. and J. M. data curation; T. T., J. M., and J. M. K. formal analysis; T. T. investigation; T. T. and J. M. K. visualization; T. T. and J. M. K. methodology; T. T., J. M., and J. M. K. writing-original draft; T. T., J. M. T., and J. M. K. writing-review and editing; T. H. and J. M. K. conceptualization; T. H. resources; T. H. and J. M. K. supervision; T. H. and J. M. K. funding acquisition.

Funding and additional information—This work was supported by National Institutes of Health Grant R01GM134000 (to J. M. K.). T. T. was supported by National Institutes of Health Grant T32CA099110, and National Institutes of Health Grant R35GM122569 (to T. H.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: GPCR, G protein-coupled receptor; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DGS-NTA(Ni), 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypropyl)-iminodiacetic acid)succinyl] nickel salt; MST2-FL, MST2 full-length; MST2-KL, MST2 kinase and linker domain; MST2-K, MST2 kinase domain; TCEP, tris(2-carboxyethyl)phosphine; YFP, yellow fluorescent protein; mYFP, monomeric yellow fluorescent protein; TIRF, total internal reflection microscopy; DAPI, 4′,6-diamidino-2-phenylindole; IPTG, isopropyl β-d-1-thio-galactopyranoside; FKBP, FK506-binding protein; FRB, FKBP-rapamycin-binding domain; SiMPull, single-molecule pulldown analysis; TEV, tobacco etch virus; MBP, maltose-binding protein; OD, optical density.

References

1. Boggiano, J. C., and Fehon, R. G. (2012) Growth control by committee: intercellular junctions, cell polarity, and the cytoskeleton regulate Hippo signaling. Dev. Cell 22, 695–702 CrossRef Medline
2. Karaman, R., and Halder, G. (2018) Cell junctions in Hippo signaling. Cold Spring Harbor Perspect. Biol. 10, a028753 CrossRef Medline
3. Misra, J. R., and Irvine, K. D. (2018) The Hippo signaling network and its biological functions. Annu. Rev. Genet. 52, 65–87 CrossRef Medline
4. Zheng, Y., and Pan, D. (2019) The Hippo signaling pathway in development and disease. Dev. Cell 50, 264–282 CrossRef Medline
5. Davis, J. R., and Tapon, N. (2019) Hippo signaling during development. Development 146, dev167106 CrossRef Medline
6. Moya, I. M., and Halder, G. (2019) Hippo–YAP/TAZ signaling in organ regeneration and regenerative medicine. Nat. Rev. Mol. Cell Biol. 20, 211–226 CrossRef Medline
7. Dey, A., Varelas, X., and Guan, K.-L. (2020) Targeting the Hippo pathway in cancer, fibrosis, wound healing and regenerative medicine. Nat. Rev. Drug Discov. 19, 480–494 CrossRef Medline
8. Wu, S., Huang, J., Dong, J., and Pan, D. (2003) hippo Encodes a Ste-20 Family Protein Kinase that restricts cell proliferation and promotes apoptosis in conjunction with salivador and warts. Cell 114, 445–456 CrossRef Medline
9. Lai, Z.-C., Wei, X., Shimizu, T., Ramos, E., Rohrbaugh, M., Nikolaidis, N., Ho, L.-L., and Li, Y. (2005) Control of cell proliferation and apoptosis by Mob as tumor suppressor. Mols. Cell 20, 675–685 CrossRef Medline
10. Huang, J., Wu, S., Barrera, J., Matthews, K., and Pan, D. (2005) The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila homolog of YAP. Cell 122, 421–434 CrossRef Medline
11. Justice, R. W., Zilian, O., Woods, D. F., Noll, M., and Bryant, P. J. (1995) The Drosophila tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. Genes Dev. 9, 534–546 CrossRef Medline
12. Xu, T., Wang, W., Zhang, S., Stewart, R. A., and Yu, W. (1995) Identifying tumor suppressors in genetic mosaics: the Drosophila lats gene encodes a putative protein kinase. Development 121, 1053–1063 CrossRef Medline
13. Tapon, N., Harvey, K. F., Bell, D. W., Wahrer, D. C. R., Schirpo, T. A., Haber, D. A., and Hariharan, I. K. (2002) salivador promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. Cell 110, 467–478 CrossRef Medline
14. Kango-Singh, M., Nolo, R., Tao, C., Verstreken, P., Hiesinger, P. R., Bellen, H. J., and Halder, G. (2002) Sharp-e mediates cell proliferation arrest during imaginal disc growth in Drosophila. Development 129, 5719–5730 CrossRef Medline
15. Harvey, K. F., Pfleger, C. M., and Hariharan, I. K. (2003) The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. Cell 114, 457–467 CrossRef Medline
16. Udan, S. R., Kango-Singh, M., Nol, R., Tao, C., and Halder, G. (2003) Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. Nat. Cell Biol. 5, 914–920 CrossRef Medline
17. Camargo, F. D., Gokhale, S., Johnnidis, J. B., Fu, D., Bell, G. W., Jaenisch, R., and Brummelkamp, T. R. (2007) YAP1 increases organ size and expands undifferentiated progenitor cells. Curr. Biol. 17, 2054–2060 CrossRef Medline
18. Dong, J., Feldmann, G., Huang, J., Wu, S., Zhang, N., Comerford, S. A., Gayyed, M. F., Anders, R. A., Maitra, A., and Pan, D. (2007) Elucidation of a universal size-control mechanism in Drosophila and mammals. Cell 130, 1120–1133 CrossRef Medline
19. Overholtzer, M., Zhang, J., Smolen, G. A., Muir, B., Li, W., Sgroi, D. C., Deng, C.-X., Brugge, J. S., and Haber, D. A. (2006) Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 ampiclon. Proc. Natl. Acad. Sci. U. S. A. 103, 12405–12410 CrossRef Medline
20. McClatchey, A. I., and Giovannini, M. (2005) Membrane organization and tumorigenesis—the NF2 tumor suppressor, Merlin. Genes Dev. 19, 2265–2277 CrossRef Medline
21. Meng, Z., Moroishi, T., and Guan, K.-L. (2016) Mechanisms of Hippo pathway regulation. Genes Dev. 30, 1–17 CrossRef Medline
22. Zhang, L., Ren, F., Zhang, Q., Chen, Y., Wang, B., and Jiang, J. (2008) The TEAD/TEF family of transcription factor Scalloped mediates Hippo signaling in organ size control. Dev. Cell 14, 377–387 CrossRef Medline
23. Wu, J., Li, W., Craddock, B. P., Foreman, K. W., Mulvhill, M. J., Qi, Q.-S., Miller, W. T., and Hubbard, S. R. (2008) Small-molecule inhibition and activation-loop trans-phosphorylation of the IGFI receptor. EMBO J. 27, 1985–1994 CrossRef Medline
EDITORS’ PICK: Increasing proximity triggers MST2 autophosphorylation

24. Goulev, Y., Fauny, J. D., Gonzalez-Marti, B., Flagiello, D., Silber, J., and Zider, A. (2008) SCALLOPED interacts with YORKIE, the nuclear effector of the Hippo tumor-suppressor pathway in Drosophila. *Curr. Biol.* 18, 435–441 CrossRef Medline

25. Oh, H., and Irvine, K. D. (2009) In vivo regulation of Yorkie phosphorylation and localization. *Development* 135, 1081–1088 CrossRef Medline

26. Oh, H., and Irvine, K. D. (2009) In vivo analysis of Yorkie phosphorylation sites. 28, 1916–1927 CrossRef Medline

27. Chan, E. H. Y., Nousiainen, M., Chalamalasetty, R. B., Schäfer, A., Nigg, E. A., and Silljé, H. H. W. (2005) The Ste20-like kinase Mst2 activates the human large tumor suppressor kinase Lats1. *Oncogene* 24, 2076–2086 CrossRef Medline

28. Millward, T. A., Hess, D., and Hemmings, B. A. (1999) Ndr protein kinase is regulated by phosphorylation on two conserved sequence motifs. *J. Biol. Chem.* 274, 33847–33850 CrossRef Medline

29. Stegert, M. R., Hergovich, A., Tamaskovic, R., Bichsel, S. J., and Hemmings, B. A. (2005) Regulation of NDR protein kinase by hydrophobic motif phosphorylation mediated by the mammalian Ste20-like kinase MST3. *Mol. Cell Biol.* 25, 11019–11029 CrossRef Medline

30. Jin, Y., Dong, L., Lu, Y., Wu, W., Hao, Q., Zhou, Z., Jiang, J., Zhao, Y., and Pan, D. (2010) Kibra functions as a tumor suppressor protein that regulates Hippo signaling in conjunction with Merlin and Expanded. *Dev. Cell* 18, 288–299 CrossRef Medline

31. Yu, J., Zheng, Y., Dong, J., Klusza, S., Deng, W.-M., and Pan, D. (2010) Kibra suppressor expanded function together in the cell membrane to control tissue growth and organ size in Drosophila. *Dev. Biol. Dev. Biol.* 337, 274–283 CrossRef Medline

32. Deng, Y., Matsui, Y., Zhang, Y., and Lai, Z.-C. (2013) Hippo activation is regulated by phosphorylation on two conserved sequence motifs. *Mol. Cell* 5509 CrossRef Medline

33. Glantschnig, H., Rodan, G. A., and Reszka, A. A. (2002) Mapping of MST1 kinase sites of phosphorylation: activation and autophosphorylation. *J. Biol. Chem.* 277, 42987–42996 CrossRef Medline

34. Praskova, M., Khoklatchev, A., Ortiz-Vega, S., and Avruch, J. (2004) Regulation of the MST1 kinase by autophosphorylation, by the growth inhibitory proteins, RASSFI and NOREI, and by Ras. *Biochem. J.* 381, 453–462 CrossRef Medline

35. Boggiano, J. C., Vanderzalm, P. J., and Fehon, R. G. (2011) Tao-1 phosphorylates Hippo/MST kinases to regulate the Hippo-Salvador-Warts tumor suppressor pathway. *Dev. Cell* 21, 888–895 CrossRef Medline

36. Poon, C. L. C., Lin, J. I., Zhang, X., and Harvey, K. F. (2011) The sterile 20-like kinase Tao-1 controls tissue growth by regulating the Salvador-Warts-Hippo pathway. *Dev. Cell* 21, 896–906 CrossRef Medline

37. Plouffe, S. W., Meng, Z., Lin, K. C., Lin, B., Hong, A. W., Chun, J. V., and Guan, K.-L. (2016) Characterization of Hippo pathway components by gene inactivation. *Mol. Cell* 64, 993–1008 CrossRef Medline

38. Creasy, C. L., Ambrose, D. M., and Chernoff, J. (1996) The Ste20-like protein kinase, MST1, dimerizes and contains an inhibitory domain. *J. Biol. Chem.* 271, 21049–21053 CrossRef Medline

39. Jin, Y., Dong, L., Lu, Y., Wu, W., Hao, Q., Zhou, Z., Jiang, J., Zhao, Y., and Zhang, L. (2012) Dimerization and cytoplasmic localization regulate Hippo kinase signaling activity in organ size control. *J. Biol. Chem.* 287, 5784–5796 CrossRef Medline

40. Bae, S. J., and Luo, X. (2018) Activation mechanisms of the Hippo kinase with its regulation by RASSF5. *C. R. Acad. Sci. Paris, Ser. II B* 345, 50–58 CrossRef Medline

41. McCartney, B. M., Kulikauskas, R. M., LaJeunesse, D. R., and Fehon, R. G. (1999) Increasing proximity triggers MST2 autophosphorylation. *J. Biol. Chem.* 274, 4264–4276 CrossRef Medline

42. Bae, S. J., Ni, L., Osisnki, A., Tomichcik, D. R., Brautigam, C. A., and Luo, X. (2017) SAV1 promotes Hippo kinase activation through antagonizing the PP2A phosphate STRIPAK. *eLife* e03278 CrossRef Medline

43. Cairns, L., Tran, T., Fowl, B. H., Patterson, A., Kim, Y. J., Bothner, B., and Kavran, J. M. (2018) Salvador has an extended SARAH domain that mediates binding to Hippo kinase. *J. Biol. Chem.* 293, 5532–5543 CrossRef Medline

44. Cairns, L., Patterson, A., Weingartner, K. A., Koehler, T. J., DeAngelis, D. R., Tripp, K. W., Bothner, B., and Kavran, J. M. (2017) The spectrin cytoskeleton regulates the Hippo signaling pathway. *EMBO J.* 36, 940–954 CrossRef Medline

45. Callus, B. A., Verhagen, A. M., and Vaux, D. L. (2006) Association of mammalian sterile twenty kinases, Mst1 and Mst2, with Salvador via C-terminal coiled-coil domains, leads to its stabilization and phosphorylation. *FEBS J.* 273, 4264–4276 CrossRef Medline

46. Jain, A., Liu, R., Ramani, B., Arauz, E., Ishitsuka, Y., Ragunathan, K., Park, J., Chen, X., Yang, Y. K., and Ha, T. (2011) Probing cellular protein complexes by single-molecule pull-down. *Nature* 473, 484–488 CrossRef Medline

47. Jain, A., Arauz, E., Aggarwal, V., Ikon, N., Chen, J., and Ha, T. (2014) Stoichiometry and assembly of mTOR complexes revealed by single-molecule
Editors' Pick: Increasing proximity triggers MST2 autophosphorylation

64. Shront, A. L., Montefusco, D. J., and Weis, R. M. (2003) Template-directed assembly of receptor signaling complexes. *Biochemistry 42*, 13379–13385

65. Zhang, X., Gureasko, J., Shen, K., Cole, P. A., and Kuriyan, J. (2006) An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. *Cell 125*, 1137–1149

66. Castellano, F., Montcourrier, P., Guillomet, J.-C., Gouin, E., Machesky, L., Cossart, P., and Chavrier, P. (1999) Inducible recruitment of Cdc42 or WASP to a cell-surface receptor triggers actin polymerization and filopodium formation. *Curr. Biol. 9*, 351–361

67. Graef, I. A., Holsinger, L. J., Diver, S., Schreiber, S. L., and Crabtree, G. R. (1997) Proximity and orientation underlie signaling by the non-receptor tyrosine kinase ZAP70. *EMBO J. 16*, 5618–5628

68. Inoue, T., Heo, W. D., Grimley, J. S., Wandleess, T. J., and Meyer, T. (2005) An inducible translocation strategy to rapidly activate and inhibit small GTPase signaling pathways. *Nat. Methods 2*, 415–418

69. Ohsushi, S., Güntert, P., Koshiba, S., Tomizawa, T., Akasaka, R., Tochio, N., Sato, M., Inoue, M., Harada, T., Watanabe, S., Tanaka, A., Shirouzu, M., Kigawa, T., and Yokoyama, S. (2007) Solution structure of an atypical WW domain in a novel β-clam-like dimeric form. *FEBS Lett. 581*, 462–468

70. Record, C. J., Chaiuadi, A., Rellos, P., Das, S., Pike, A. C. W., Fedorov, O., Marsden, B. D., Knapp, S., and Lee, W. H. (2010) Structural comparison of human mammalian Ste20-like kinases. *PLoS One 5*, e11905

71. Ig, C., and Pi, C. (2005) Coincidence detection in phosphoinositide signaling. *Trends Cell Biol. 15*, 540–547

72. Kholodenko, B. N., Hoek, J. B., and Westerhoff, H. V. (2000) Why cytoplasmic signaling proteins should be recruited to cell membranes. *Trends Cell Biol. 10*, 173–178

73. McLaughlin, S., Wang, J., Gambhir, A., and Murray, D. (2002) PIP 2 and proteins: interactions, organization, and information flow. *Annu. Rev. Biophys. Biomol. Struct. 31*, 151–175

74. Groves, J. T., and Kuriyan, J. (2010) Molecular mechanisms in signal transduction at the membrane. *Nat. Struct. Mol. Biol. 17*, 659–665

75. Ubersax, J. A., and Ferrell, J. E. (2007) Mechanisms of specificity in protein phosphorylation. *Nat. Rev. Mol. Cell Biol. 8*, 530–541

76. Lin, Z., Xie, R., Guan, K., and Zhang, M. (2020) A WW tandem-mediated dimerization mode of SAV1 essential for Hippo signaling. *Cell Rep. 32*, 10818

77. Lemmon, M. A., Freed, D. M., Schlessinger, J., and Kiyatkin, A. (2016) The dark side of cell signaling: positive roles for negative regulators. *Cell 164*, 1172–1184

78. Xu, Q., Malecka, K. L., Fink, L., Jordan, E. J., Duffy, E., Kolander, S., Peterson, J. R., and Dunbrack, R. L. (2015) Identifying three-dimensional structures of autophosphorylation complexes in crystals of protein kinases. *Sci. Signal. 8*, rs13–rs13

79. Lamontanara, A. J., Georgeon, S., Trias, G., Svergun, D. I., and Hantschel, O. (2014) The SH2 domain of Ab1 kinases regulates kinase autophosphorylation by controlling activation loop accessibility. *Nat. Commun. 5*, 1–11

80. Shinohara, H., Behar, M., Inoue, K., Hiroshima, M., Yasuda, T., Nagashima, T., Kimura, S., Sanjo, H., Maeda, S., Yunomoto, N., Ki, S., Akira, S., Sako, Y., Hoffmann, A., Kurosaki, T., et al. (2014) Positive feedback within a kinase signaling complex functions as a switch mechanism for NF-κB activation. *Science 344*, 760–764

81. Chung, J. K., Nocka, L. M., Decker, A., Wang, Q., Kadlecnek, T. A., Weiss, A., Kuriyan, J., and Groves, J. T. (2019) Switch-like activation of Bruton’s tyrosine kinase by membrane-mediated dimerization. *Proc. Natl. Acad. Sci. U. S. A. 116*, 10798–10803

82. Zhou, H.-X. (2004) Polymer models of protein stability, folding, and interactions. *Biochemistry 43*, 2141–2154

83. Sørensen, C. S., Jendroszek, A., and Kjaergaard, M. (2019) Linker dependence of avidity in multivalent interactions between disordered proteins. *J. Mol. Biol. 431*, 4784–4795

84. Freed, D. M., Bessman, N. J., Kiyatkin, A., Salazar-Cavazos, E., Byrne, P. O., Moore, J. O., Valley, C. C., Ferguson, K. M., Leahy, D. J., Lidke, D. S., and Lemmon, M. A. (2017) EGFR ligands differentially stabilize receptor dimers to specify signaling kinetics. *Cell 171*, 683–695

85. Furemi, S. G. B., Liang, Y.-L., Nowell, C. J., Hollis, M. L., Woolsey, P. J., Maso, E. D., Inoue, A., Christopoulos, A., Wootten, D., and Sexton, P. M. (2016) Ligand-dependent modulation of G protein conformation alters drug efficacy. *Cell 167*, 739–746

86. Herenbrink, C. K., Sykes, D. A., Donthamsetti, P., Canals, M., Coudrat, T., Shonberg, J., Scammells, P. J., Capuano, B., Sexton, P. M., Charlton, S. I., Javitch, J. A., Christopoulos, A., and Lane, J. R. (2019) The role of kinase context in apparent biased agonism at GPCRs. *Nat. Commun. 10*, 2959

87. Lane, J. R., May, L. T., Parton, R. G., Sexton, P. M., and Christopoulos, A. (2017) A kinetic view of GPCR allostery and biased agonism. *Nat. Chem. 9*, 929–937

88. Wacker, D., Stevens, R. C., and Roth, B. L. (2017) How ligands illuminate GPCR molecular pharmacology. *Cell 170*, 414–427

89. Rawat, S. J., Araiza-Olivera, D., Arias-Romero, L. E., Villamar-Cruz, O., Prudnikova, T. Y., Roder, H., and Chernoff, J. (2016) H-ras inhibits the Hippo pathway by promoting Mst1/Mst2 heterodimerization. *Curr. Biol. 26*, 1556–1563

90. Polesello, C., Huelsmann, S., Brown, N. H., and Tapon, N. (2006) The Drosophila RASSF homolog antagonizes the Hippo pathway. *Curr. Biol. 16*, 2459–2465

91. Cordenonsi, M., Zanconato, F., Azzolin, L., Forcato, M., Rosato, A., Frassetto, C., Inui, M., Montagner, M., Parenti, A. R., Poletti, A., Daidone, M. G., Dupont, S., Basso, G., Biccio, S., and Piccolo, S. (2011) The Hippo transducer TAZ confers cancer stem cell–related traits on breast cancer cells. *Cell 147*, 759–772

92. Cai, D., Feliciano, D., Dong, P., Flores, E., Gruebele, M., Porat-Shliom, N., Sukenik, S., Liu, Z., and Lippincott-Schwartz, J. (2019) Phase separation of Yap reorganizes genome topology for long-term Yap target gene expression. *Nat. Cell Biol. 21*, 1578–1589

93. Peränen, J., Rikkonen, M., Hyvönen, M., and Kääriäinen, L. (1996) T7 vector: a versatile and efficient recombinant lambda prophage for large-scale gene expression. *J. Bacteriol. 178*, 3731–3737

94. Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to Image: 25 years of image analysis. *Nat Methods 9*, 671–675

95. Tu-Seckin, B., and Raben, D. M. (2012) Dual regulation of diacylglycerol kinase (DGK)–β: polybasic proteins promote activation by phospholipids and increase substrate affinity. *J. Biol. Chem. 287*, 41619–41627

96. Longo, P. A., Kavan, J. M., Kim, M.-S., and Leahy, D. J. (2013) Transient mammalian cell transfection with polyethyleneimine (PEI). *Methods Enzymol. 529*, 227–240

97. Roy, R., Hohng, S., and Ha, T. (2008) A practical guide to single-molecule FRET. *Nat. Methods 5*, 507–516
Increasing kinase domain proximity promotes MST2 autophosphorylation during Hippo signaling

Thao Tran, Jaba Mitra, Taekjip Ha and Jennifer M. Kavran

*J. Biol. Chem.*, 2020, 295:16166-16179.
doi: 10.1074/jbc.RA120.015723 originally published online September 29, 2020

Access the most updated version of this article at doi: 10.1074/jbc.RA120.015723

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 97 references, 27 of which can be accessed free at http://www.jbc.org/content/295/47/16166.full.html#ref-list-1