Hippo pathway signaling limits cell growth and proliferation and maintains the stem-cell niche. These cellular events result from the coordinated activity of a core kinase cassette that is regulated, in part, by interactions involving Hippo, Salvador, and dRassF. These interactions are mediated by a conserved coiled-coil domain, termed SARAH, in each of these proteins. SARAH domain–mediated homodimerization of Hippo kinase leads to autophosphorylation and activation. Paradoxically, SARAH domain–mediated heterodimerization between Hippo and Salvador enhances Hippo kinase activity in cells, whereas complex formation with dRassF inhibits it. To better understand the mechanism by which each complex distinctly modulates Hippo kinase and pathway activity, here we biophysically characterized the entire suite of SARAH domain–mediated complexes. We purified the three SARAH domains from Drosophila melanogaster and performed an unbiased pulldown assay to identify all possible interactions, revealing that isolated SARAH domains are sufficient to recapitulate the cellular assemblies and that Hippo is a universal binding partner. Additionally, we found that the Salvador SARAH domain homodimerizes and demonstrate that this interaction is conserved in Salvador’s mammalian homolog. Using native MS, we show that each of these complexes is dimeric in solution. We also measured the stability of each SARAH domain complex, finding that despite similarities at both the sequence and structural levels, SARAH domain complexes differ in stability. The identity, stoichiometry, and stability of these interactions characterized here comprehensively reveal the nature of SARAH domain–mediated complex formation and provide mechanistic insights into how SARAH domain–mediated interactions influence Hippo pathway activity.

The Hippo pathway controls tissue growth and differentiation by regulating the rate of cell proliferation and apoptosis (1–6). Gene expression is silenced upon phosphorylation and cytoplasmic retention of the transcriptional co-factor Yorkie (1, 3–8). Aberrant pathway activity leads to overgrowth phenotypes in both mammalian and fly models and is associated with tumorigenesis in humans (1, 3–11). Hippo signal transduction relies on the activity of a core kinase cassette that includes two kinases, Hippo and Warts, and two accessory proteins, Salvador and Mats. These proteins, together with Yorkie, form a core kinase cassette that is responsible for gene regulation. Each component of this core cassette has a mammalian homolog with similar function. The mechanisms regulating the activity of this kinase cassette are also most likely conserved as disruption of the genes encoding either Hippo (hpo) or Mats (mts) in flies results in overgrowth phenotypes that can be rescued by complementation with the mammalian genes encoding the homologous proteins, Mst1/2 or Mob1, respectively (5, 7, 10).

The activity of the core cassette begins with the activation of Hippo kinase, or Mst1/2 in mammals, which involves phosphorylation of the activation loop (12, 13). Genetic, cellular, and in vitro data suggest that homodimerization mediated by the conserved C-terminal coiled-coil domain, termed SARAH, of either Hippo or Mst1/2 promotes autophosphorylation in trans and kinase activation (6, 14–19). Activated Hippo then phosphorylates Warts. This phosphorylation event leads to Warts activation, which also involves autoprophosphorylation and complex formation with Mats (5, 20–22). Warts kinase can then phosphorylate the transcriptional co-factor Yorkie, leading to the accumulation of phosphorylated Yorkie in the cytoplasm (7, 8).

SARAH domains were first identified as a conserved coiled-coil region located at the C termini of three members of the Hippo pathway, Salvador, dRassF, and Hippo, and were named after each (23). Dimeric, trimeric, and tetrameric assemblies of coiled-coils have been proposed, but the preponderance of evidence supports a dimeric assembly with limited evidence for tetrameric assemblies and no evidence for trimeric ones (14, 18, 23–34). Structural studies have each revealed a conserved arrangement of two antiparallel coiled-coils with each mono-
mer adopting a short 3/10-helix followed by a turn and a long α-helix (18, 25–32). The dimer interface is mediated by interstrand interactions along the length of each α-helix and provides a rationale for why each SARAH domain–mediated complex is mutually exclusive. The SARAH domain of Salvador contains an extended N-terminal region that forms an additional α-helix that enlarges the dimer interface with Hippo (31, 32), and Salvador variants lacking this extension bind less tightly to Hippo SARAH domain (32). Together, these data suggest that Hippo:Salvador complexes are tighter than other SARAH domain–mediated complexes. Analysis of the interactions of SARAH domain complexes have largely focused on the mammalian homologs of Hippo and dRassF with little to no data for Salvador SARAH domain, and reported dissociation constants vary widely, from nanomolar to millimolar (29, 30, 35–37). There has been no systematic study of the entire set of possible SARAH domain–mediated interactions, making it difficult to understand the likelihood of any given complex forming.

Salvador and dRassF bind Hippo via their respective SARAH domains and yet have opposing roles in regulating the activity of Hippo kinase, with Salvador stimulating pathway activity and dRassF inhibiting it (3, 24). The mechanisms by which SARAH domain–mediated complex formation influences Hippo kinase activity are still a matter of investigation. The importance of these SARAH domain proteins to Hippo pathway activity is underscored by the observation that flies bearing alleles encoding variants of either Hippo or Salvador that lack SARAH domains display overgrowth phenotypes typical of pathway disruption (4, 6). Likewise, combination of mutant alleles of dRassF and Hippo lacking SARAH domains leads to a stronger overgrowth phenotype than either allele separately (24). Each SARAH domain–mediated complex correlates with a distinct phosphorylation state of Hippo. Immunoprecipitation assays revealed that unphosphorylated Hippo bound dRassF and phosphorylated Hippo bound Salvador (24). Evidence suggests two possible routes for the inhibition of the pathway by dRassF. Complex formation between Hippo and dRassF would prevent homodimerization of Hippo and thus block autophosphorylation and thus activation. dRassF could further promote the unphosphorylated state of Hippo by acting as a scaffold to recruit the phosphatase-containing complex dSTRIPAK to Hippo (38). The functions of the mammalian dRassF homologs are more complex, showing either inactivation or activation of Mst1/2, depending on cell type and the level of Hippo pathway activity (16, 39). Co-expression of the human homolog of Salvador, hSalvador, with Mst1/2 leads to higher levels of activation loop phosphorylation (5, 31, 32). The increase in phosphorylation of Mst1/2 can be partly attributed to hSalvador inhibiting the phosphatase activity of the STRIPAK complex (31) but does not address which molecular event promotes the initial phosphorylation of Hippo. Interpretation of existing cellular and genetic data are complicated by an incomplete understanding of how the biophysical characteristics, such as stoichiometry and stability, of each SARAH domain–mediated complex are different. Because these parameters influence the likelihood of complex formation, they also influence the activity of the pathway.

To rationalize the function of SARAH domains in modulating Hippo pathway activity, we undertook a systematic study of the biophysical parameters of complex formation by the entire suite of isolated SARAH domain complexes. We chose to work with the Drosophila SARAH domains because they are a simpler system than their mammalian counterparts; there is only one homolog of each Drosophila SARAH domain compared with multiple isoforms of the human homologs of Hippo and dRassF. We began by purifying each SARAH domain individually to systematically study the entire set of SARAH domain complexes. We report here in vitro binding studies that revealed that a previously unidentified interaction between Salvador SARAH domains that we determine is dimeric, mediates association of the full-length protein in cells, is less stable than other SARAH domain interactions, and is conserved in hSalvador. We also find that the binding patterns of isolated SARAH domains recapitulate those previously observed in cells; the specificity of these interactions, therefore, is derived from the SARAH domains and not from other protein domains or cellular factors. We also report biophysical analysis of SARAH domain interactions and show that each of the interactions is dimeric in solution, but they have varying stabilities. Our results provide biophysical constraints that define the nature of SARAH domain–mediated complex formation and have implications for the molecular mechanisms of these complexes on pathway activity.

Results

The Hippo SARAH domain is a universal binding partner

We previously determined the domain boundaries for the Drosophila melanogaster Salvador SARAH domain (32), which then enabled us to purify each of the three SARAH domains from D. melanogaster, Salvador, Hippo, and dRassF (Fig. 1). To determine which complexes could be formed by isolated SARAH domains, we performed an unbiased in vitro pulldown assay using purified proteins. Untagged SARAH domains were
incubated with hexahistidine (H6)² and thioredoxin-tagged SARAH domains immobilized on nickel-affinity resin; because the SARAH domains have similar mobility on SDS-PAGE, the thioredoxin tag was added to change the migration of one SARAH domain in each pulldown to help with visualization. The resin was collected and washed, and the amount of untagged SARAH domain bound was monitored by Coomassie-stained SDS-PAGE. Hippo SARAH domain bound each SARAH domain tested, Hippo, Salvador, and dRassF. In contrast, Salvador SARAH domain and dRassF SARAH domain bound only themselves or Hippo SARAH domain. No interactions were detected between Salvador and dRassF SARAH domains, regardless of which protein was the bait or prey in the experiment. To ensure complexes were homogeneous in future assays, SARAH domain heterocomplexes were co-expressed and purified using tandem-affinity purification. Because we observed an interaction between Salvador SARAH domains in the pulldown assay (Fig. 2), we asked whether this SARAH domain also mediated homooligomerization of the full-length (FL) protein in cells. Because hSalvador homodimerizes through an atypical WW domain (40, 41), binding between differentially tagged FL proteins was used as a positive control. Binding between a FL and a variant containing the isolated SARAH domain was used to detect SARAH domain–mediated homooligomerization. HEK293 cells were transiently co-transfected with plasmids encoding FLAG-tagged Salvador FL and variants corresponding to either Salvador FL or the SARAH domain tagged with the HA epitope and monomeric yellow fluorescent protein (mYFP). Complexes were isolated from cell lysates by immunoprecipitation using an anti-HA antibody, and complex formation was monitored by Western blotting. FLAG-tagged Salvador FL co-immunoprecipitated with both HA-mYFP Salvador FL and HA-mYFP Salvador SARAH domain. These interactions demonstrate that the SARAH domain of Salvador is sufficient to mediate homooligomerization in cells. To determine whether this interaction is conserved across species, we performed an analogous set of co-immunoprecipitation experiments with hSalvador (Fig. 3B). The HA-mYFP–tagged SARAH domain of hSalvador was able to immunoprecipitate FLAG-tagged hSalvador FL, indicating that homooligomer-

²The abbreviations used are: H6, hexahistidine; IMAC, immobilized metal affinity chromatography; FL, full-length; mYFP, monomeric yellow fluorescent protein; Kᵣ, equilibrium constant of unfolding; N, native; U, unfolded; ΔGᵤ, Gibbs free energy of unfolding; ΔGₚH₂O, Gibbs free energy in water; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; MRE, molar residue ellipticity; R, universal gas constant; T, temperature; yᵪₒ, observed signal; yᵪₑ, signal from native protein; Fᵪₑ, fraction native; yᵦₑ, signal from unfolded protein; Fₑᵤ, fraction unfolded; aᵪ, y intercept for the native protein; aᵦ, y intercept for the unfolded protein; bᵪ, slope of the baseline for the native region of the sigmoid; bₑ, slope of the baseline for the native region of the sigmoid; Pₑ, total concentration of protein; HA, hemagglutinin; SUMO, small ubiquitin-like modifier.
mediated by the SARAH domain of Salvador is conserved across species.

**SARAH domains are dimeric**

We next wanted to determine the stoichiometry of each of the five possible SARAH domain complexes identified (Hippo: Hippo, Salvador: Salvador, dRassF: dRassF, Hippo: Salvador, and Hippo: dRassF) (Fig. 1). We choose to employ native MS as it analyzes the assembly of complexes in physiological buffers without the addition of exogenous tags or chemical cross-linkers that may either disrupt physiologically relevant complexes or stabilize nonphysiologically relevant ones. Each complex was analyzed by electrospray ionization MS under native and denaturing conditions (Fig. 4 and Fig. S1). For each spectrum, the m/z values of the observed peaks were deconvoluted to the molecular weight, and the identity of the complex was assigned based on those weights (Table 1).

The highest oligomeric species detected under native conditions for each of the five complexes were dimers—homodimers in the case of Hippo, Salvador, and dRassF SARAH domains and heterodimers for complexes between Hippo and either Salvador or dRassF SARAH domains. Under denaturing conditions, only monomeric species were detected for the Hippo, dRassF, or Hippo:dRassF SARAH domain samples. The charge state distribution of the monomeric peaks was at a lower m/z (higher charge) than those in the native spectra, suggesting that the denaturing buffer reduced the stability of the monomeric SARAH domains. In contrast, peaks corresponding to either homodimers of Salvador SARAH domain or heterodimers of Hippo: Salvador SARAH domains were maintained under denaturing conditions and had the same charge state distribution as in the native conditions, suggesting that homo- and heterodimers containing Salvador SARAH domain were not disrupted by the denaturing buffer. Most likely the hydrophobic
interactions mediated by the N-terminal extension of the Salvador SARAH domain (32) are stabilized under denaturing conditions.

**SARAH domain dimers have varying stabilities**

To understand the likelihood of each SARAH domain-mediated complex forming, we determined the relative stabilities of each using chemically induced equilibrium unfolding. For SARAH domains, complex formation and domain unfolding are coupled; dissociation of homodimers resulted in unfolded monomeric proteins for homodimers of both Mst1 and a mammalian dRassF homolog as well as a heterodimer between the two (29, 30, 35). Therefore, the fraction of folded protein corresponds to the number of complexes formed, and the equilibrium constant of unfolding ($K_u$) varies according to the dissociation constant ($K_d$) (42).

For each of the five SARAH domain complexes, we performed titrations at three different protein concentrations (Fig. 4). For each titration, we ensured that equilibrium conditions were satisfied for each point and that unfolding curves corresponding to denaturing and renaturing experiments were superposable. In each case, the unfolding curve was sigmoidal with a single transition, implying that the unfolding reaction involved a simple two-state transition; SARAH domains went from folded to unfolded without populating any significant intermediate conformations. At low urea concentrations, the unfolding curves for both Salvador and dRassF SARAH domains did reveal a possible second transition. However, in both cases, the data corresponding to a possible intermediate represented a sufficiently small fraction of the system that an intermediate state could not be accurately modeled.

To determine the thermodynamic constants that describe the stability of each SARAH domain dimer, we fit the data to a two-state model of unfolding in which a native folded dimer ($N_2$) transitions to two unfolded monomers ($2U$), $N_2 \rightleftharpoons 2U$.

$K_u$ can be expressed in terms of the ratio of concentrations of unfolded and folded protein, $K_u = [U]^2/[N_2]$, or in terms of free energy of unfolding ($\Delta G_u$), $K_u = \exp(-\Delta G_u/RT)$. $\Delta G_u$ can be linearly extrapolated to determine the Gibbs free energy in the absence of urea ($\Delta G_{u293}$) for each complex, which has the advantage of allowing for comparison of stabilities between different proteins without being influenced by the denaturant (43). For each of the five SARAH domain complexes, we performed titrations at three different protein concentrations (Fig.
The midpoint of the transition for each unfolding curve varied with protein concentration, demonstrating that the observed transitions corresponded to the disassociation of the dimers. Therefore, to accurately determine the thermodynamic parameters, data from three titrations performed at different protein concentrations were fit simultaneously using nonlinear least-squares fitting and global analysis (Table 2 and Fig. 5).

The stabilities of the SARAH domain complexes varied by nearly 10 kcal/mol (Table 2). The Salvador:Hippo SARAH domain complex was the most stable, with a ΔG_H2O of 17.7 ± 0.4 kcal/mol and a K_u of 0.1 μM, whereas the Salvador SARAH domain homodimer was the least stable, with a ΔG_H2O of 7.8 ± 0.4 kcal/mol and a K_u of 1.8 μM. Hippo, dRassF, and the Hippo: dRassF SARAH domain dimers had similar, intermediate stabilities with ΔG_H2O values of 11.9 ± 0.2, 13.3 ± 0.3, and 13.5 ± 0.2 kcal/mol and K_u values of 1.9, 0.17, or 0.12 nM, respectively.

The m-values, which correspond to the surface area exposed upon unfolding (44), follow a similar pattern as the ΔG_H2O and K_u values. Hippo:Hippo, dRassF:dRassF, and Hippo:dRassF SARAH domain complexes have similar m values, Hippo: Salvador SARAH domains the largest, and Salvador SARAH domain the smallest.

Discussion

Our ability to purify each Drosophila SARAH domain protein allowed us to investigate the biophysical nature of complex formation for the entire suite of SARAH domains and enabled us to directly determine and compare key biophysical parameters among all possible protein complexes (Fig. 1). We started by performing an unbiased pulldown assay using purified SARAH domains to determine which complexes are mediated by isolated SARAH domains. Our results show that the Hippo
Biophysical characterization of SARAH domains

Table 2

| Protein complex                     | m value \( ^{\text{a}} \) (kcal/mol/s) | \( \Delta G_{\text{H2O}} \) \( ^{\text{a}} \) (kcal/mol) | \( K_u \) (M) |
|-------------------------------------|----------------------------------------|-------------------------------------------------|---------------|
| Salvador SARAH domain homodimer     | 0.6 ± 0.04                             | 7.8 ± 0.4                                    | 1.8 × 10^{-6} |
| Hippo SARAH domain homodimer        | 1.6 ± 0.05                             | 11.9 ± 0.2                                   | 1.9 × 10^{-9} |
| dRassF SARAH domain homodimer       | 1.5 ± 0.07                             | 13.3 ± 0.3                                   | 1.7 × 10^{-10}|
| Hippo:dRassF SARAH domain heterodimer | 1.6 ± 0.04                           | 13.5 ± 0.2                                   | 1.2 × 10^{-10}|
| Hippo:Salvador SARAH domain heterodimer | 2.0 ± 0.07                           | 17.7 ± 0.4                                   | 1.0 × 10^{-13}|

\( ^{\text{a}} \) Reported uncertainties represent the S.E. of the fit.

SARAH domain is a universal binding partner, interacting with itself as well as the SARAH domains from dRassF and Salvador. We found that the binding patterns observed in cells can be recapitulated using isolated SARAH domains, implying that specificity arises from the SARAH domains and not additional factors, such as other protein domains or co-factors (Fig. 2). These binding experiments also revealed an unanticipated complex, a homooligomer of the Salvador SARAH domain that we determine also mediates homooligomerization of FL protein in cells in both the fly and human homologs (Figs. 2 and 3). Using native MS, and therefore in solution and free from the addition of any chemical cross-linkers, we determined the stoichiometry of each of the five possible SARAH domain complexes, as identified in our pulldown assay. We show that each complex is dimeric and find no evidence for higher-order complexes (Fig. 4 and Table 1). The stoichiometries determined here agree with those assigned to homologous mammalian complexes, demonstrating that the organization of SARAH domain complexes is conserved (14, 18, 25–32).

The complexes mediated by the SARAH domain of Hippo, both with itself as well as with the SARAH domains of Salvador and dRassF, influence the activation state of Hippo and, in turn, overall pathway activity. As each possible complex is mutually exclusive, there will be competition between homo- and heterodimer formation (24). To rationalize the effects of each SARAH domain complex on Hippo signal transduction, we need to understand the likelihood of complex formation. We determined the stabilities of each of the five SARAH domain complexes using chemically induced equilibrium unfolding (Fig. 5 and Table 2). We observed that the midpoints of the transitions in the unfolding curves were dependent on the concentration of the SARAH domains. This dependence confirmed that disassociation of SARAH domain dimers resulted in unfolded monomers and that differences in stabilities directly correlate with differences in affinity (29, 30, 35, 42) (Fig. 5). Because the midpoint of the transitions varied with protein concentration, to accurately determine the thermodynamic parameters associated with unfolding, we needed to globally fit data from a set of unfolding curves collected at multiple protein concentrations. Each of the five possible SARAH domain complexes were fit to a two-state model of unfolding (\( N_2 = 2U \)) (Table 2), reflecting both the single transition of each unfolding curve and the stoichiometries determined by native MS.

Despite having relatively similar structures, the stabilities of the five SARAH domain complexes vary widely, over 10 kcal/mol. The Hippo:Salvador SARAH domain heterodimer is the most stable (\( \Delta G_{\text{H2O}} = 17.7 \pm 0.4 \) kcal/mol) and twice as stable as the weakest complex, the Salvador SARAH domain homo-dimer (\( \Delta G_{\text{H2O}} = 7.8 \pm 0.4 \) kcal/mol). The increased stability of the Hippo:Salvador SARAH domain heterodimer is consistent with the complex having a larger buried surface area than other SARAH domain dimers and supports the observation that, by extending the binding interface with Hippo, the N-terminal extension of the Salvador SARAH domain leads to a tighter complex (32). Other factors may also contribute to this elevated stability, such as a partially unfolded intermediate, which was observed for the Mst1 SARAH domain (35). The remaining SARAH domain complexes, Hippo:Hippo, dRassF:dRassF, and Hippo:dRassF, cluster between these two extremes with stabilities that vary only by \( ~1.5 \) kcal/mol. The stabilities of these three SARAH domain complexes also align with the relative sizes of the buried surface areas observed in the structures of their mammalian homologs (32). Because the rank orders of both the m values and \( \Delta G_{\text{H2O}} \) of the Drosophila SARAH domain complexes match the rank order of buried surface areas observed in the structures of the mammalian homologs (32), the hierarchy of stabilities of SARAH domain complexes is most likely conserved between the Drosophila and mammalian homologs.

The varying stabilities of SARAH domain complexes provide insight into the likelihood of their formation, which, in turn, will have distinct effects on Hippo kinase activity. The common consensus is that SARAH domain–mediated homodimerization of Hippo leads to autophosphorylation and kinase activation, yet the stability of the Hippo homodimer (\( \Delta G_{\text{H2O}} = 11.9 \pm 0.2 \) kcal/mol) is nearly 6 kcal/mol lower than the heterodimer with Salvador (\( \Delta G_{\text{H2O}} = 17.7 \pm 0.4 \) kcal/mol). These observations suggest that the presence of Salvador shifts the equilibrium from Hippo homodimers toward Hippo:Salvador heterodimers. Given the difference in stabilities, how would Hippo kinase initially become phosphorylated in the presence of Salvador? Phosphorylation by an upstream kinase, Tao-1, could represent one possible route of Hippo activation. However, inactivation of the TAO kinase genes in mammalian cells does not fully block Hippo pathway activity, indicating that this upstream kinase is not fully responsible for activation of Mst1/2 (45–47). These results coupled with our analysis of SARAH domain stabilities suggest that there may still be additional cellular events that promote activation of Hippo kinase. The stability of the Hippo homodimer is 1 kcal/mol lower than the heterodimer with dRassF (\( \Delta G_{\text{H2O}} = 13.3 \pm 0.3 \) kcal/mol), a negative effector of Hippo activation (24). This difference in stability provides a thermodynamic explanation for the inhibitory effects of dRassF. The presence of dRassF would shift the equilibrium toward dRassF:Hippo heterodimers and away from Hippo homodimers that can undergo autophosphorylation.
Biophysical characterization of SARAH domains

Because the Salvador:Hippo SARAH domain heterodimer is the most stable, perhaps arising from the expanded binding interface mediated by the N-terminal extension of the Salvador SARAH domain (32), it is perplexing that the Salvador SARAH domain homodimer, which contains two N-terminal extensions, is not the most stable complex; instead, the Salvador homodimer is the weakest. To understand this phenomenon, we first compared the structure of the Salvador SARAH domain homodimer, which contains two N-terminal extensions, a second copy of the Salvador SARAH domain onto the equivalent region of the Hippo-SARAH domain so that intrastand interactions along the coiled-coil were maintained (Fig. 6B). This superposition resulted in steric clashes between the N-terminal extension of one monomer and C-terminal end of the opposing monomer, rendering this arrangement implausible. We also performed a second superposition in which we aligned the C terminus of Salvador SARAH domain onto the equivalent region of Hippo so that the packing with the N-terminal extension from the other SARAH domain in the dimer would be maintained (Fig. 6C). This superposition resulted in the two α-helices becoming splayed apart at the opposite end of the coiled-coil. In this arrangement, only a fraction of the typical interstrand interactions could form. This arrangement is consistent with both the lower stability and smaller m value observed for the Salvador SARAH domain homodimer (Table 2). Despite this lower stability, the Salvador SARAH domain homodimer is sufficiently tight to be detected in both in vitro and cell-binding experiments (Figs. 2 and 3). We show that this interaction is conserved between flies and mammals (Fig. 3), but the biological implications of Salvador having two points of homodimerization, the SARAH domain and the atypical WW domain, is not yet apparent (40, 41).

Early studies established the link between SARAH domain–mediated interactions and pathway activity, but how these interactions modulate Hippo kinase activity is still a topic of investigation. Our work provides the biophysical constraints, in the form of specificity, stoichiometry, and stability, for the entire suite of SARAH domain complexes that are necessary to formulate a mechanistic framework for the interplay between SARAH domain complexes. The results presented here provide a mechanistic rationale for how dRassF inhibits Hippo kinase activation. However, these results also raise questions as to the mechanism governing Hippo kinase autophosphorylation in the presence of Salvador and suggest that there may be additional routes to the activation of Hippo kinase. The next step in understanding the regulation of the Hippo kinase cassette will be interpreting how these coiled-coil interactions function within the context of full-length proteins and multicomponent, physiological assemblies.

Experimental procedures

Protein expression and purification

Nucleotides encoding the SARAH domains of D. melanogaster sav (residues 530–608), hpo (residues 606–662), and dRassF (residues 754–802) were each cloned into a pBAD4 vector derivative downstream from a H6 and SUMO tag (49). Proteins were overexpressed in Escherichia coli and purified as described previously (32). Briefly, proteins were isolated using nickel-charged IMAC, the SUMO tag was removed by incubation with a SUMO-specific protease (SENP) at 4 °C overnight,
and the protein was further purified by anion-exchange and gel-filtration chromatographies.

For protein complexes, genes encoding the Salvador:Hippo SARAH domains and dRassF:Hippo SARAH domains were cloned into pRSF-Duet Vector (EMD-Millipore) as SUMO-tagged fusions with an additional SBP tag for Hippo and an additional H6 tag for either dRassF or Salvador. Proteins were isolated by sequential affinity purification and IMAC followed by Streptactin (GE Healthcare) and were further purified as described previously (32). All SARAH domain proteins were flash-frozen in 10 mM Tris, pH 8, 200 mM NaCl, 1 mM Tris(2-carboxylethyl)phosphine hydrochloride (TCEP).

For proteins expressed as fusions to thioredoxin, genes encoding the SARAH domains of *D. melanogaster* sav (residues 530–608), hpo (residues 606–662), or dRassF (residues 754–802) were cloned into a derivative of the pBAD4 vector downstream from H6 and thioredoxin tags (49). T7 Express cells (New England BioLabs) transformed with the plasmids encoding either Hippo or dRassF SARAH domains were grown at 37 °C, and protein expression was induced at 0.5 mM isopropyl-D-1-thiogalactopyranoside (at 37 °C for 3 h). Rosetta 2 (DE3) (Novagen) cells transformed with a vector encoding Salvador SARAH domain were grown at 37 °C, and protein expression was induced at 0.5 A_{600} with 0.5 mM isopropyl β-D-1-thiogalactopyranoside at 20 °C for 16 h. Cells were lysed in 40 mM Tris, pH 8, and 400 mM NaCl, 0.1% Nonidet P-40 and supplemented with protease inhibitor mixture (Sigma). For Salvador SARAH domain, the lysis buffer was further supplemented with 10% glycerol. The thioredoxin fusion proteins were purified by sequential IMAC and anion- and gel-filtration chromatographies. Final proteins were stored in 10 mM Tris, pH 8, 200 mM NaCl, 1 mM TCEP and flash-frozen in liquid nitrogen at 10 mg/ml.

**In vitro pulldown experiments**

25-μl reactions containing a 100 μM concentration of the indicated untagged SARAH domains and a 25 μM concentration of the indicated H6-thioredoxin–tagged SARAH domain in 10 mM Tris, pH 8.0, 200 mM NaCl, and 1 mM TCEP were incubated for 1 h at room temperature. To promote more robust binding, binding reactions with untagged dRassF SARAH domain were modified to include 500 μM untagged dRassF SARAH domain and a reaction temperature of 30 °C. To each binding reaction, 10 μl of nickel-charged Profinity IMAC resin (Bio-Rad) was added, and the reaction was further incubated for 30 min at room temperature. The resin was collected and washed three times with 500 μl of 10 mM Tris, pH 8.0, and 200 mM NaCl. The resin was boiled in SDS-loading buffer. Samples were analyzed by SDS-PAGE stained with Coomassie Brilliant Blue. Gels were imaged using the Odyssey IR imaging system (LI-COR). Band intensities were quantified in ImageJ (50). The amount of untagged SARAH domain bound above background in each complex was calculated by subtracting the amount of untagged protein bound to the H6-thioredoxin control; to normalize between replicates, the difference was divided by the amount of untagged protein bound to H6-thioredoxin for each set. Prism software (GraphPad Software) was used to plot data and for statistical analysis. Ratios were analyzed by a paired t test.

**Co-immunoprecipitation experiments**

Nucleotides encoding the FL (residues 1–608) *D. melanogaster* sav were cloned into pcDNA 3.1 (Invitrogen) downstream from either the FLAG tag or tandem HA and mYFP (51) tags. Additionally, nucleotides corresponding to the SARAH domain (residues 530–608) from *D. melanogaster* sav were also cloned downstream of HA and mYFP tags. An equivalent set of plasmids were cloned using the nucleotides encoding either the FL (residues 1–383) or SARAH domain (residues 299–383) of SAV1 from *Homo sapiens*. HEK293T cells (ATCC) 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% CO₂. Cells were transfected with the indicated plasmids using X-tremeGENE HP DNA transfection reagent (Sigma–Aldrich) according to the manufacturer’s protocol. Cells were harvested 24 h following transfection and lysed in ice-cold lysis buffer (20 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol) supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM NaVO₄, 5 mM NaF, 2.5 mM Na₃P₂O₇, 0.1 mM β-glycerophosphate, 5 mM EDTA, Protease Inhibitor Mixture (Sigma), and Universal Nuclease (Pierce). Normalized, clarified lysates were incubated with Protein G resin and HA antibody (Roche Applied Science, lot 34502100) for 2 h at 4 °C. Resin was collected, washed with lysis buffer, and boiled in SDS-loading buffer. Samples were analyzed by Western blotting using primary antibodies that recognized the HA (Roche Applied Science, lot 34502100) or FLAG (Sigma–Aldrich, lot SLCD3990) epitopes followed by either IRDye 800CW goat anti-rat (LI-COR, lot C90122-02) or IRDye 800CW goat anti-mouse (LI-COR, lot C81106-01) secondary antibodies, respectively. Blots were scanned on an Odyssey IR imaging system (LI-COR). Bands were quantified using ImageJ (50). The relative amount of FLAG-tagged protein bound was determined by first correcting for background binding by subtracting the intensity of the FLAG-tagged protein bound in the negative control, lysates lacking HA-mYFP, from each experiment. Relative binding was then determined by calculating the ratio of FLAG-tagged protein in each lane to the amount bound by the positive control, HA-mYFP Salvador FL. Ratios were analyzed by a paired t test. Prism software (GraphPad Software) was used to plot data and for statistical analysis.

**Native mass spectrometry**

MS analysis was carried out as described previously (52) using a Waters SYNAPT G2-Si instrument. Immediately prior to analysis, purified SARAH domain complexes were buffer-exchanged into 200 mM ammonium acetate, pH 7.6, using a 3K molecular weight cutoff filter ( Pall Life Sciences). Before native analysis, SARAH domain samples were buffer-exchanged and then diluted 1:100 (v/v) into 200 mM ammonium acetate for a final concentration of each SARAH domain of 0.023 mg/ml for Hippo, 0.04 mg/ml for Salvador, 0.01 mg/ml for dRassF, 0.1 mg/ml for Hippo:Salvador, and 0.012 mg/ml for Hippo:dRassF. Concentrations were chosen to maximize the ratio of signal to noise of the spectra. Sam-
samples were then infused from in-house prepared gold-coated borosilicate glass capillaries into the electrospray source at ~90 nl/min with the following settings: source temperature 30 °C, capillary voltage 1.75 kV, argon collision cell flow rate 7 ml/min, transfer collision energy 5 V, trap collision energy 20 V. Comparisons between samples were only made in cases where settings were identical. For denatured analyses, each sample was diluted 1:10 (v/v) into 200 mM ammonium acetate, pH 7.6, and then 1:10 (v/v) into 50% methanol plus 1% formic acid or 50% methanol plus 0.5% formic acid for the Salvador SARAH domain. Denatured samples were analyzed using the same settings as used for the native analysis. Data analysis was performed using the Waters MassLynx version 4.1 software with the Maximum Entropy add-on.

**Urea-induced equilibrium denaturation**

CD data were collected with an Aviv model 420 spectropolarimeter (Lakewood, NJ) equipped with a temperature control cell holder and an automatic syringe titrator (Hamilton Microlab 500 titrator). Measurements were collected at 25 °C in a cuvette with a 1-cm path length at 222 nm and averaged over 30 s. Titrations were performed at the indicated protein concentrations, where protein concentration corresponds to molar amount of total protein chains, and the sample was equilibrated for 300 s between injections. Native samples were equilibrated in 10 mM Tris, pH 8.0, 200 mM NaCl, 1 mM TCEP (Thermo), and denatured samples were equilibrated in the same buffer supplemented with ~8 M urea (VWR). Urea was purified using mixed bed resin (Bio-Rad) (53), and the concentration of urea stock solutions was determined by refractive index (43). Three denaturing titrations and one renaturing titration were performed for each sample.

**Global fitting of denaturation data using a two-state model**

Raw CD data were converted to molar residue ellipticity (MRE) (degrees × cm²/dmol) to normalize signal arising from differences in protein concentration, and fits were performed on MRE values. SARAH domain unfolding was fit to a dimeric, two-state folding model in which a native folded dimer (Nₙ) transitions to two unfolded monomers (2U) (29, 30, 35). This process can be expressed as follows.

\[ [Nₙ] = 2[U] \]  
(Eq. 1)

\[ \Delta G_u = [U]^2 / [Nₙ] \]  
(Eq. 2)

\[ K_u = \exp (- \Delta G_u / RT) \]  
(Eq. 3)

where \( R \) is the Universal gas constant and \( T \) is the temperature in K. \( \Delta G_u \) is linearly dependent on the concentration of urea (43) and can be expressed as follows,

\[ \Delta G_u = \Delta G_{H₂O} + m [\text{urea}] \]  
(Eq. 4)

where \( \Delta G_{H₂O} \) is the change in the Gibbs free energy of the SARAH domain dimer in water and \( m \) is the slope of the urea dependence.

**Biophysical characterization of SARAH domains**

The observed signal for each data point \( \left(Y_{obs}\right) \) can be expressed as the sum of the signal \( \left(Yₙ\right) \) arising from the fraction native \( \left(Fₙ\right) \) and the signal \( \left(Y_u\right) \) from the fraction unfolded \( \left(F_u\right) \).

\[ Y_{obs} = (1 - F_u)Yₙ + F_u \times Y_u \]  
(Eq. 5)

The native and unfolded signals are linearly dependent on the concentration of urea and can be rewritten as follows,

\[ Yₙ = aₙ + bₙ [\text{urea}] \]  
(Eq. 6)

\[ Y_u = a_u + b_u [\text{urea}] \]  
(Eq. 7)

where \( aₙ \) and \( a_u \) are the y intercepts, and \( bₙ \) and \( b_u \) are the slopes of the baselines corresponding to the native and unfolded regions of the sigmoid, respectively. The total concentration of protein \( \left(P_T\right) \) is equal to the sum of the concentrations of the native and unfolded chains.

\[ \left[P_T\right] = [U] + 2[Nₙ] \]  
(Eq. 8)

Equation 8 can be rewritten as the sum of the fraction of native and unfolded chains, which equals 1.

\[ 1 = Fₙ + F_u = [U]/[P_T] + 2[Nₙ]/[P_T] \]  
(Eq. 9)

By substituting Equation 9 into Equation 2, \( F_u \) can also be expressed as follows.

\[ F_u = \left(-K_u + (K_u^2 + 8K_u)^{1/2}ight)/4[P_T] \]  
(Eq. 10)

Isolation of \( F_u \) allows for substitution of Equation 10 into Equation 5, resulting in an expression in which \( Y_{obs} \) depends on urea and which can be used to determine \( \Delta G_{H₂O} \).

Three data sets, at varying protein concentrations, for each SARAH domain were fitted globally using the nonlinear fitting function in Mathematica (Wolfram Research), constraining \( \Delta G_{H₂O} \) and \( m \), while treating \( aₙ \), \( bₙ \), \( a_u \), and \( b_u \) for each concentration as locally adjustable parameters (54).

To enable easy comparison among the sigmoids and fits from different SARAH domains, the data and fits in the figure were converted to fraction folded using the equation

\[ F_n = \left(Y_{obs} - Y_u\right) / \left(Yₙ - Y_u\right) \]  
(Eq. 11)

using \( Y_u \) and \( Yₙ \) values obtained from the fits. This normalization does result in variations in the baseline regions that were not present in the raw data.

**Structural analysis**

Superpositions were performed using least-squares fitting in CCP4 (55) and visually inspected in Coot (56). Atomic model representations were generated in PyMOL (Schrodinger, LLC).

**Data availability**

Raw MS data have been deposited in the Mass Spectrometry Interactive Virtual Environment (MassIVE) with the project identifier MSV000085022. All other data contained within the article are available upon request from the corresponding author, Jennifer Kavran (jkavran@jhu.edu).
Biophysical characterization of SARAH domains

Author contributions—L. C. and J. M. K. conceptualization; L. C., A. P., K. A. W., T. K., D. R., and K. W. T. data curation; L. C., A. P., K. A. W., T. K., B. B., and J. M. K. formal analysis; L. C., A. P., K. A. W., T. K., D. R., K. W. T., B. B., and J. M. K. writing—review and editing; L. C., A. P., T. K., and J. M. K. writing—original draft; B. B. and J. M. K. funding acquisition; B. B. and J. M. K. project administration; J. M. K. resources; J. M. K. supervision.

Acknowledgments—We thank Doug Barrick and Thao Tran for helpful scientific discussions. We thank the Johns Hopkins Center for Molecular Biophysics for providing facilities and resources. Funding for the Proteomics, Metabolomics, and Mass Spectrometry Facility at Montana State University was made possible in part by the M. J. Murdock Charitable Trust; NIGMS, National Institutes of Health, Grant P20GM103474; and the Biological and Electron Transfer and Catalysis (BETCy) EFRC funded by the United States Department of Energy, Office of Science (DE-SC0012518).

References

1. 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
2. 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 Medline
3. Tapon, N., Harvey, K. F., Bell, D. W., Wahrer, D. C. R., Schiripo, T. A., Haber, D., and Hariharan, I. K. (2002) salvador promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. Cell 110, 467–478 CrossRef Medline
4. Kango-Singh, M., Nolo, R., Tao, C., Verstreken, P., Hiesinger, P. R., Bellen, H. J., and Halder, G. (2002) Shar-pei mediates cell proliferation arrest during imaginal disc growth in Drosophila. Development 129, 5719–5730 CrossRef Medline
5. 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 salvador and warts. Cell 114, 445–456 CrossRef Medline
6. 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
7. 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
8. 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
9. St John, M. A. R., Tao, W., Fei, X., Fukumoto, R., Carcangiu, M. L., Brownstein, D. G., Parlow, A. F., McGrath, J., and Xu, T. (1999) Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction. Nat. Genet. 21, 182–186 CrossRef Medline
10. 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, Mats. Cell 120, 675–685 CrossRef Medline
11. Camargo, F. D., Gokhale, S., Johnnidas, I. 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
12. 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
13. Colombani, J., Polesello, C., Josue, F., and Tapon, N. (2006) Dmp53 activates the Hippo pathway to promote cell death in response to DNA damage. Curr. Biol. 16, 1453–1458 CrossRef Medline
14. Crespy, 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
15. Deng, Y., Pang, A., and Wang, J. H. (2003) Regulation of mammalian STE20-like kinase 2 (MST2) by protein phosphorylation/dephosphorylation and proteolysis. J. Biol. Chem. 278, 11760–11767 CrossRef Medline
16. Praskova, M., Khoklatchev, A., Ortiz-Vega, S., and Avruch, J. (2004) Regulation of the MST1 kinase by autophosphorylation, by the growth inhibitoty proteins, RASSF1 and NORE1, and by Ras. Biochem. J. 381, 453–462 CrossRef Medline
17. 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
18. Ni, L., Li, S., Yu, J., Min, J., Brautigam, C. A., Tomchick, D. R., Pan, D., and Luo, X. (2013) Structural basis for autoactivation of human Mst2 kinase and its regulation by RASSF5. Structure 21, 1757–1768 CrossRef Medline
19. Deng, Y., Matsu, Y., Zhang, Y., and Lai, Z.-C. (2007) Hippo activation through homodimerization and membrane association for growth inhibition and organ size control. Dev. Biol. 375, 152–159 CrossRef Medline
20. Chan, E. H. Y., Nousainen, M., Chalalmasletty, 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
21. 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
22. Stegert, M. R., Hergovich, A., Tamasvok, 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
23. Scheel, H., and Hofmann, K. (2003) A novel interaction motif, SARAH, connects three classes of tumor suppressor. Curr. Biol. 13, R899–R900 CrossRef Medline
24. Polesello, C., Huelsmann, S., Brown, N. H., and Tapon, N. (2006) The Drosophila RASSF homolog antagonizes the Hippo pathway. Curr. Biol. 16, 2459–2465 CrossRef Medline
25. Hwang, E., Ryu, K.-S., Pääkkönen, K., Güntert, P., Cheong, H.-K., Lim, D.-S., Lee, J.-O., Jeon, Y. H., and Cheong, C. (2007) Structural insight into dimereic interaction of the SARAH domains from Mst1 and RASSF family proteins in the apoptosis pathway. Proc. Natl. Acad. Sci. U.S.A. 104, 9236–9241 CrossRef Medline
26. Chaikuad, A., Krojer, T., Newman, J. A., Dixon-Clarke, S., von Delft, F., Arrowsmith, C. H., Edwards, A. M., Bountra, C., Knapp, S., and Structural Genomics Consortium (SGC) (2013) Crystal structure of STK3 (MST2) SARAH domain. Protein Data Bank. PDB ID: 4LON
27. Liu, G. G., Shi, Z. B., and Zhou, Z. C. (2013) Crystal structure of human MST2 SARAH domain. Protein Data Bank. PDB ID: 4HKD
28. Chaikuad, A., Krojer, T., Kopec, J., von Delft, F., Arrowsmith, C. H., Edwards, A. M., Bountra, C., and Structural Genomics Consortium (SGC) (2013) Crystal structure of STK4 (MST1) SARAH domain. Protein Data Bank. PDB ID: 4NR2
29. Hwang, E., Cheong, H.-K., Mustaq, A. U., Kim, H.-Y., Yeo, K. J., Kim, E., Lee, W. C., Hwang, K. Y., Cheong, C., and Jeon, Y. H. (2014) Structural basis of the heterodimerization of the MST and RASSF SARAH domains in the Hippo signalling pathway. Acta Crystallogr. D Biol. Crystallogr. 70, 1944–1953 CrossRef Medline
30. Makbul, C., Constantinescu Aruxandei, D., Hofmann, E., Schwarz, D., Wolf, E., and Herrmann, C. (2013) Structural and thermodynamic characterization of Nore1-SARAH: a small, helical module important in signal transduction networks. Biochemistry 52, 1045–1054 CrossRef Medline
Biophysical characterization of SARAH domains

31. Bae, S. J., Ni, L., Osinski, A., Tomchick, D. R., Brautigam, C. A., and Luo, X. (2017) SAV1 promotes Hippo kinase activation through antagonizing the PP2A phosphatase STRIPAK. *eLife* 6, e30278 CrossRef Medline

32. 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

33. Hauri, S., Wepf, A., van Drogen, A., Varjosalo, M., Tapon, N., Aebersold, R., and Gstaiger, M. (2013) Interaction proteome of human Hippo signaling: modular control of the co-activator YAP1. *Mol. Syst. Biol.* 9, 713 CrossRef Medline

34. Sánchez-Sanz, G., Matallanas, D., Nguyen, L. K., Khodolenko, B. N., Rosta, E., Kolch, W., and Buchete, N.-V. (2016) MST2-RASSF protein-protein interactions through SARAH domains. *Brief. Bioinform.* 17, 593–602 CrossRef Medline

35. Constantinescu Aruxandei, D., Makbul, C., Koturenkiene, A., Lüdemann, M.-B., and Herrmann, C. (2011) Dimerization-induced folding of MST1 SARAH and the influence of the intrinsically unstructured inhibitory domain: low thermodynamic stability of monomer. *Biochemistry* 50, 10990–11000 CrossRef Medline

36. Margineanu, A., Chan, J. I., Kelly, D. J., Warren, S. C., Flatten, D., Kumar, S., Katan, M., Dunsby, C. W., and French, P. M. W. (2016) Screening for protein-protein interactions using Förster resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy (FLIM). *Sci. Rep.* 6, 28186 CrossRef Medline

37. Bitra, A., Sistla, S., Mariam, J., Malvi, H., and Anand, R. (2017) Rassf proteins as modulators of Mst1 kinase activity. *Sci. Rep.* 7, 45020 CrossRef Medline

38. Ribeiro, P. S., Josué, F., Wepf, A., Wehr, M. C., Rinner, O., Kelly, G., Tapon, N., and Gstaiger, M. (2010) Combined functional genomic and proteomic approaches identify a PP2A complex as a negative regulator of Hippo signaling. *Mol. Cell* 39, 521–534 CrossRef Medline

39. Khokhlatchev, A., Rabizadeh, S., Xavier, R., Nedwidek, M., Chen, T., Zhang, X.-F., Seed, B., and Avruch, J. (2002) Identification of a novel Ras-regulated proapoptotic pathway. *Curr. Biol.* 12, 253–265 CrossRef Medline

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

41. Ohnishi, 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-clamp-like dimeric form. *FEBS Lett.* 581, 462–468 CrossRef Medline

42. Mok, Y. K., de Prat Gay, G., Butler, P. J., and Bycroft, M. (1996) Equilibrium dissociation and unfolding of the dimeric human papillomavirus strain-16 E2 DNA-binding domain. *Protein Sci.* 5, 310–319 CrossRef Medline

43. Pace, C. N. (1986) Determination and analysis of urea and guanidine hydrochloride denaturation curves. *Methods Enzymol.* 131, 266–280 CrossRef Medline

44. Street, T. O., Courtmanche, N., and Barrick, D. (2008) Protein folding and stability using denaturants. *Methods Cell Biol.* 84, 295–325 CrossRef Medline

45. Poon, C. L. C., Lin, J. L., 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

46. 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

47. 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

48. Szimler, T., Grácz, É., Györfy, D., Végh, B., Szilágyi, A., Hajdú, L., Závodszky, P., and Vas, M. (2019) New type of interaction between the SARAH domain of the tumour suppressor RASSF1A and its mitotic kinase Aurora A. *Sci. Rep.* 9, 5550 CrossRef Medline

49. Peränen, J., Rikkonen, M., Hyvönen, M., and Kääriäinen, L. (1996) T7 vectors with modified T7lac promoter for expression of proteins in *Escherichia coli*. *Anal. Biochem.* 236, 371–373 CrossRef Medline

50. 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 CrossRef Medline

51. Jain, A., Arauz, E., Sagarwal, V., Ikon, N., Chen, J., and Ha, T. (2014) Stoichiometry and assembly of mTOR complexes revealed by single-molecule pulldown. *Proc. Natl. Acad. Sci. U.S.A.* 111, 17833–17838 CrossRef Medline

52. Luo, M. L., Jackson, R. N., Denny, S. R., Tokmina-Lukaszewska, M., Mak-simchuk, K. R., Lin, W., Bothner, B., Wiedenheft, B., and Beisel, C. L. (2016) The CRISPR RNA-guided surveillance complex in *Escherichia coli* accommodates extended RNA spacers. *Nucleic Acids Res.* 44, 7385–7394 CrossRef Medline

53. Wingfield, P. T. (2001) Use of protein folding reagents. *Curr. Protoc. Protein Sci.* Appendix 3, Appendix 3A CrossRef Medline

54. Gloss, L. M., and Matthews, C. R. (1997) Urea and thermal equilibrium denaturation studies on the dimerization domain of the tumour suppressor RASSF1A and its mitotic kinase Aurora A. *Acta Crystallogr. D Biol. Crystallogr.* 53, 521–534 CrossRef Medline

55. Pera, N., Rizzo, M., and Fratelli, F. (2019) Use of protein folding reagents. *Curr. Protoc. Protein Sci.* Appendix 3, Appendix 3A CrossRef Medline

56. Wingfield, P. T. (2001) Use of protein folding reagents. *Curr. Protoc. Protein Sci.* Appendix 3, Appendix 3A CrossRef Medline

57. Simonsen, K., and Frattini, P. (2019) Use of protein folding reagents. *Curr. Protoc. Protein Sci.* Appendix 3, Appendix 3A CrossRef Medline

58. Gross, L. M., and Matthews, C. R. (1997) Urea and thermal equilibrium denaturation studies on the dimerization domain of the tumour suppressor RASSF1A and its mitotic kinase Aurora A. *Acta Crystallogr. D Biol. Crystallogr.* 53, 521–534 CrossRef Medline

59. Pera, N., Rizzo, M., and Fratelli, F. (2019) Use of protein folding reagents. *Curr. Protoc. Protein Sci.* Appendix 3, Appendix 3A CrossRef Medline

60. Wingfield, P. T. (2001) Use of protein folding reagents. *Curr. Protoc. Protein Sci.* Appendix 3, Appendix 3A CrossRef Medline

61. Simonsen, K., and Frattini, P. (2019) Use of protein folding reagents. *Curr. Protoc. Protein Sci.* Appendix 3, Appendix 3A CrossRef Medline