

University of Massachusetts Medical School
eScholarship@UMMS

Open Access Articles

Open Access Publications by UMMS Authors

2018-09-28


Angiotensins stimulate LATS kinase autophosphorylation and act as scaffolds that promote Hippo signaling

Sebastian Mana-Capelli
University of Massachusetts Medical School

Et al.

Let us know how access to this document benefits you.

Follow this and additional works at: <https://escholarship.umassmed.edu/oapubs>

 Part of the [Amino Acids, Peptides, and Proteins Commons](#), [Biochemistry Commons](#), [Cell Biology Commons](#), [Cells Commons](#), and the [Enzymes and Coenzymes Commons](#)

Repository Citation

Mana-Capelli S, McCollum D. (2018). Angiotensins stimulate LATS kinase autophosphorylation and act as scaffolds that promote Hippo signaling. Open Access Articles. <https://doi.org/10.1074/jbc.RA118.004187>. Retrieved from <https://escholarship.umassmed.edu/oapubs/3602>

This material is brought to you by eScholarship@UMMS. It has been accepted for inclusion in Open Access Articles by an authorized administrator of eScholarship@UMMS. For more information, please contact Lisa.Palmer@umassmed.edu.

Angiomotins stimulate LATS kinase autophosphorylation and act as scaffolds that promote Hippo signaling

Sebastian Mana-Capelli, and Dannel McCollum*

Department of Biochemistry & Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA, 01605

Running Title: Activation of LATS1/2 by AMOT

*Address correspondence to: Dannel McCollum, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA, 01605. Tel.: (508) 856-8767. E-mail: dannel.mccollum@umassmed.edu

Keywords: AMOT, angiomotin, LATS, MOB1, YAP, Hippo pathway, SAV1, MST, scaffold protein, cell signaling

ABSTRACT

The Hippo pathway controls cell proliferation, differentiation, and survival by regulating the YAP transcriptional coactivator in response to various stimuli, including the mechanical environment. The major YAP regulators are the LATS1/2 kinases, which phosphorylate and inhibit YAP. LATS1/2 are activated by phosphorylation on a hydrophobic motif (HM) outside the kinase domain by MST1/2 and other kinases. Phosphorylation of the HM motif then triggers autophosphorylation of the kinase in the activation loop (AL) to fully activate the kinase, a process facilitated by MOB1. The angiomin family of proteins (AMOT, AMOTL1, and AMOTL2) bind LATS1/2 and promote its kinase activity and YAP phosphorylation through an unknown mechanism. Here, we show that angiominins increase Hippo signaling through multiple mechanisms. We found that by binding LATS1/2, SAV1, and YAP, angiominins function as a scaffold that connects LATS1/2 to both its activator SAV1–MST1 and its target YAP. Deletion of all three angiominins reduced the association of LATS1 with SAV1–MST1 and decreased MST1/2-mediated LATS1/2-HM phosphorylation. Angiominin deletion also reduced LATS1/2's ability to associate with and phosphorylate YAP. In addition, we found that angiominins have an unexpected function along with MOB1 to promote autophosphorylation of LATS1/2 on the AL motif independent of HM phosphorylation. These results indicate that angiominins enhance Hippo signaling by stimulating LATS1/2 autophosphorylation and by connecting LATS1/2 with both its activator SAV1–MST1/2 and its substrate YAP.

Introduction

The Hippo pathway regulates the transcriptional coactivators YAP and TAZ (hereafter referred to as YAP) to control cell fate decisions regarding cell proliferation, survival, and differentiation (1). Mis-regulation of the Hippo-YAP pathway is commonly associated with cancer (2). The LATS1 and LATS2 kinases (LATS1/2) are major regulators of YAP. LATS1/2 phosphorylate YAP to trigger its cytoplasmic retention and degradation (3-5). In the canonical Hippo pathway the MST1 and MST2 kinases

(MST1/2) activate LATS1/2 (6). LATS1/2 can also be activated by MAP4K-family and TAO kinases (7-9). All upstream kinases phosphorylate LATS1/2 on a conserved hydrophobic motif (HM: T1079 and T1041 in LATS1 and LATS2 respectively). LATS1/2-HM phosphorylation stimulates it to autophosphorylate in the activation loop (AL: S909 and S872 in LATS1 and LATS2 respectively) and become fully active (6,10,11). This process is facilitated by the MOB1 protein, which enhances LATS1/2-HM phosphorylation by MST1/2 and stimulates LATS1/2-AL autophosphorylation (10-12). MST1/2 phosphorylation of LATS1/2 is also stimulated by colocalization of both proteins on the membrane, which is promoted by SAV1 and NF2 respectively (13). In addition, SAV1 can function as a scaffold since it can bind both MST1/2 and LATS1/2 (14,15). Although there are numerous other proteins that can promote Hippo signaling, their mechanism of action is in general not well understood.

A major regulator of Hippo signaling is the mechanical environment, which includes factors such as cell density, substrate stiffness, and cell stretch or mechanical tension. How these mechanical stimuli act through the core Hippo regulators to control YAP activity is not clear. Several studies have shown that the actin cytoskeleton, which responds dynamically to mechanical changes, is a major regulator of Hippo signaling (16-20). Reduction in F-actin activates LATS1/2. How F-actin controls LATS1/2 activity is not certain, but may involve angiominins (AMOT, AMOTL1, and AMOTL2) (21-23). Angiominins bind to both LATS1/2 and YAP, and can inhibit YAP through two mechanisms; 1) binding and retention of YAP in the cytoplasm/plasma membrane, and 2) activation of LATS1/2 (21,24-27). Other studies have shown that AMOT can control YAP nuclear/cytoplasmic localization, depending on its phosphorylation state (28). Angiominins also bind the LATS1/2 activator NF2 (29,30), and a study using proximity-labeling found that SAV1 and MOB1 may interact with AMOT (31). We and others have proposed that angiominins may act as scaffolds to promote LATS1/2 activation. A conserved N-terminal region in the long form

of AMOT (AMOT130, hereafter referred to as AMOT) contains an F-actin binding motif, and flanking L/PPxY sites that bind WW-containing proteins (such as YAP). We have shown previously that F-actin and YAP compete for binding to angiomotins (22), which may provide one mechanism for how F-actin can influence YAP activity; when F-actin levels go down, angiomotins are free to bind and inhibit YAP in the cytoplasm and possibly activate LATS1/2. Angiomotins are required for relocalization of YAP from the nucleus to the cytoplasm when F-actin is disrupted (22). In addition, elimination of angiomin regulation of YAP causes patterning defects in early mouse embryos (26,27), transformation of MDCK cells (25), and epithelial to mesenchymal transition in MCF10 cells (32). How angiomotins activate LATS1/2 is not known. Here we show that the angiomin protein AMOT interacts with multiple core Hippo pathway regulators to stimulate LATS1/2 to phosphorylate YAP via three distinct mechanisms. First, AMOT promotes interaction between LATS1/2 and its activators MST1/2 and SAV1. Second AMOT enhances LATS1/2 phosphorylation of YAP by promoting interaction between the two proteins. And third, AMOT collaborates with MOB1 to enhance LATS1/2-AL autophosphorylation, even in the absence LATS-HM site phosphorylation.

Results

Angiomotins promote LATS1/2 activation

Phosphorylation at the LATS2-HM site is presumed to be the primary regulatory site for LATS1/2 because, when phosphorylated, it promotes autophosphorylation in the activation loop (AL) of the kinase to make it fully active (6,10,11), allowing it to phosphorylate YAP and other substrates. We tested the effect of angiomotins on phosphorylation at the LATS1/2-HM site. Overexpression of all three angiomotins (AMOT, AMOTL1, and AMOTL2) promoted LATS2-HM phosphorylation (Figure 1A). (Note that in this study all experiments utilize the long form of AMOT (AMOT130)). Consistent with these observations, deletion of all 3 angiomin genes (Amot-3KO) in HEK293 cells using CRISPR (Figure S1A-B) resulted in reduced activating phosphorylation of LATS1/2 at the HM and AL sites after F-actin disruption

by Latrunculin B (Lat B) treatment (Figure 1B). Similar results were observed in HeLa cells with all 3 angiomotins knocked down using siRNA (Figure S1C). Consistent with our findings, other studies showed that knockdown of angiomotins reduces LATS1/2 activity, as judged by reduced YAP S127 phosphorylation in HEK293T, MDCK, and MCF10A cells (21,25,32). Together these findings show that angiomotins can activate LATS1/2 by promoting phosphorylation at the HM site in response to F-actin perturbation.

To better understand how angiomotins activate LATS1/2, we tested which core Hippo components are required for angiomotins to promote HM phosphorylation. We found that in HEK293 cells, MST1/2 are the primary LATS1/2-HM kinases since deletion of MST1/2 (Figure S1D) causes a major decrease in HM phosphorylation in unperturbed, Lat B, and okadaic acid (OA) treated cells (Figure S1E-F). Consistent with this observation, the increase LATS2-HM phosphorylation after angiomin overexpression was largely lost in MST1/2 deleted cells (Figure 1A), suggesting that angiomin stimulated LATS2-HM phosphorylation works primarily through MST1/2 in HEK293 cells. Similar results were observed in the absence of SAV1 and NF2 (Figure 1C; S1D), the MST1/2 and LATS1/2 binding partners respectively. Note that not even the phospho-mimetic (active) form of AMOT (AMOT-175E) is able to activate LATS1/2 in the absence of SAV1 or NF2. These results are consistent with the idea that angiomotins may act together with the two kinase modules (MST-SAV1 and LATS1/2-NF2) to promote LATS1/2 activation.

MST1/2 and SAV1 stimulate AMOT-LATS2 binding

While examining how MST1/2, SAV1, and NF2 contribute to angiomin activation of LATS1/2, we discovered a potential positive feedback loop whereby MST1/2 and SAV1 promote AMOT-LATS2 binding. When we tested whether MST1/2, SAV1, and NF2 affect AMOT-LATS2 binding, we observed that deletion of MST1/2 or SAV1 greatly reduced binding of AMOT to LATS2 (NF2 deletion reduced binding but the

effect did not reach the level of significance) (Figure 1D-E; see S1G for further antibody controls). One explanation for these results could be that AMOT-LATS2 binding requires LATS2 activity, and MST1/2 and SAV1 are required for LATS2 activation. This would be consistent with our previous study showing that AMOTL2 bound better to wild-type than kinase dead LATS2, or LATS2 with both sites of activating phosphorylation mutated to alanine residues (21). We tested whether AMOT had similar binding preferences. This experiment showed that AMOT, like AMOTL2, bound preferentially to wild-type LATS2 compared to LATS2 with both sites of activating phosphorylation mutated (Figure 2A). Because LATS2 is known to phosphorylate AMOT on S175 (22,26,33-35), these results could be explained if either LATS2 phosphorylation of AMOT promotes AMOT-LATS2 binding, or if AMOT preferentially binds to the active form of LATS2. Consistent with the first model, we observed, as seen previously (26), that a phospho-mimetic version of AMOT (AMOT-175E) binds to (Figure 2B) and activates LATS2 (Figure S2A) better than the non-phosphorylatable form of AMOT (AMOT-175A). However, because expression of AMOT-175E did not rescue the AMOT-LATS2 binding defect in MST1/2-KO cells, additional factors must contribute to the AMOT-LATS2 binding defect in MST1/2-KO cells (Figure 2C). Therefore, we tested the second model; namely that angiomotins bind preferentially to active LATS2. If correct, then expression of a phospho-mimetic version of LATS2 (LATS2-1041E) should rescue the defect in AMOT-LATS2 interaction in MST1/2 deleted cells. Indeed, this turned out to be the case (Figure 2D). These results show that MST1/2 and SAV1 activation of LATS1/2 stimulates a positive feedback mechanism by promoting AMOT-LATS1/2 binding.

AMOT is a Hippo pathway scaffold protein

AMOT could activate LATS1/2 either by stimulating MST1/2 activation, or by promoting the ability of MST1/2 to phosphorylate LATS1/2. Our results support the later possibility since AMOT overexpression did not affect the levels of MST1/2 activating

phosphorylation (Figure S2B). (Note that to remove potential issues with feedback from LATS1/2, we conducted these and subsequent experiments with AMOT-175E.) We next investigated whether AMOT might function as a scaffold to bring LATS1/2 together with its activator MST1/2 and substrate YAP. This and previous studies showed that AMOT binds LATS1/2 (21,36). Thus one potential mechanism of action for AMOT could be that it connects LATS1/2 with the MST1/2-SAV1 complex and YAP. Indeed, when we analyzed endogenous AMOT complexes using chromosomally tagged sfGFP-MAP-AMOT (37) in HEK293 cells (created using CRISPR mediated genome modification), we could detect LATS1, SAV1, MST1, and YAP coming down with sfGFP-MAP-AMOT isolated using streptavidin beads (note that part of the MAP tag is a streptavidin binding peptide) (Figure 3A), consistent with endogenous AMOT associating with core Hippo pathway proteins. We next examined whether AMOT might contribute to association of core Hippo pathway proteins by overexpressing AMOT with different combinations of Hippo pathway proteins followed by immunoprecipitation. Since AMOT bound LATS1/2, we tested whether AMOT could stimulate LATS2 activation by also binding MST1. While we were unable to observe binding between AMOT and MST1 when both proteins were co-expressed (Figure 3B), AMOT did bind to SAV1 in a manner that depended on the SAV1 WW domains and the 3 L/PPxY motifs (WW domain ligands) in AMOT (Figure 3C). SAV1 expression allowed MST1 to come down in AMOT immune complexes (Figure 3B) indicating that SAV1 could bridge interaction between AMOT and MST1. These results support the notion that AMOT could connect LATS2 to SAV1-MST1. However, as previously observed in *Drosophila* (14), SAV1 was also able to bind LATS2 (Figure 3D)(presumably by interaction of its WW domain with the PPxY motif in LATS2), showing that it can act as a scaffold for MST1 and LATS2 without AMOT (at least when over-expressed). Interestingly, the amount of SAV1 that came down in LATS2 immune complexes was enhanced by expression of AMOT (Figure 3D). Similar experiments were also done to

examine how the interaction of MST1 with LATS2 might depend on SAV1 and AMOT (Figure 3E). MST1 and LATS2 were found to not interact unless SAV1 was co-expressed (Figure 3E). As with the SAV1-LATS2 interaction (Figure 3D), AMOT alone could not promote interaction between LATS2 and MST1, but was able to enhance the interaction of MST1 and SAV1 with LATS2 (Figure 3E). Together these results suggest that AMOT may enhance the assembly/stabilization of a complex between SAV1-MST1 and LATS2. In addition, AMOT may also act as a scaffold to bring LATS1/2 in proximity with its substrate YAP. We observed that when YAP and LATS2 were co-expressed, YAP was only poorly recovered in LATS2 immune complexes (Figure 3F) unless AMOT was also expressed. The ability of AMOT to enhance LATS2-YAP binding depended on the L/PPxY motifs in AMOT (Figure 3F). This result suggested that YAP binding by AMOT might not just inhibit YAP by sequestering it in the cytoplasm as previously reported, but might also bring it together with its inhibitory kinase LATS1/2. Consistent with this model, Amot-3KO cells show defects in YAP phosphorylation (Figure S3A) and are defective in keeping YAP out of the nucleus after growth to high density or serum deprivation (Figure S3B). We also saw defects in association of endogenous core Hippo pathway proteins in the absence of angiomotins. Whereas immunoprecipitates of endogenous LATS1 from HEK293 cells contained SAV1 and YAP, both proteins were almost completely absent in LATS1 immunoprecipitates from Amot-3KO cells (Figure 4A). Similarly, when we immunoprecipitated endogenous SAV1 from HEK293 cells, we could detect MST1, LATS1, AMOT, and YAP in the immune complexes. However SAV1 immune complexes from Amot-3KO cells contained MST1, but almost no YAP and LATS1 (Figure 4B). This indicates that at endogenous protein levels, SAV1 alone cannot efficiently scaffold LATS1/2 with MST1/2. Collectively these results show that AMOT may regulate LATS1/2 and YAP at least in part by acting as a scaffold to connect LATS1/2 to both its activator SAV1-MST1/2, as well as its target YAP.

Angiomotins promote LATS1/2-AL auto-phosphorylation

The ability of AMOT to promote a complex containing SAV1, MST1, and LATS2 could explain why LATS1/2 activating phosphorylation at the HM site (the site phosphorylated by MST1/2) is reduced in Amot-3KO cells response to Lat B treatment (Figure 1B). Similarly, we observed that LATS1/2-HM phosphorylation is reduced in Amot-3KO cells compared to wild-type when MST1 is overexpressed (Figure 5A). The defect in LATS2-HM phosphorylation in Amot-3KO cells in response to MST1 overexpression could be rescued by co-overexpression of SAV1 (Figure 5B), consistent with SAV1 being able to replace the scaffolding function of angiomotins, at least when overexpressed. Interestingly, although Amot-3KO cells overexpressing MST1 and SAV1 had HM phosphorylation levels similar to wild-type controls expressing MST1 and SAV1, they still had a defect in LATS2 phosphorylation at the AL site (Figure 5B). These results raised the possibility that angiomotins may promote LATS2 autophosphorylation at the LATS2-AL site, independent their effect on MST1/2 phosphorylation at the LATS2-HM site. (Note that the defect in LATS2-AL phosphorylation in Amot-3KO cells could be rescued by re-expression of the 3 angiomin protein (Figure S1B)). To distinguish between direct effects of AMOT on LATS2-AL phosphorylation and indirect effects via LATS2-HM phosphorylation, we tested whether AMOT could promote AL phosphorylation of a version of LATS2 that mimicked the HM phosphorylated form (LATS2-1041E) but could not be regulated by MST1/2 (Figure S4A) or other upstream HM kinases. We used LATS2-1041E because we assumed that AMOT alone might not be able promote LATS2-AL phosphorylation without HM phosphorylation. We found that AMOT expression enhanced AL phosphorylation of LATS2-1041E (Figure 5C). Thus, AMOT can promote LATS2 auto-phosphorylation at the AL site independent of any effects it has on LATS2-HM phosphorylation. The only other protein known to stimulate LATS1/2 auto-phosphorylation is MOB1 (10,12). We observed that when co-expressed (Figure S4B), MOB1A could be detected in AMOT immune complexes,

showing that the two proteins might interact. Therefore, we investigated the relationship between MOB1 and AMOT in promoting LATS2-AL phosphorylation. Several lines of evidence suggested that MOB1 may act independently from angiomotins and the effects of AMOT on LATS1/2-AL phosphorylation may depend on MOB1. For example, MOB1A binding to LATS2 was not affected by deletion of all three angiomotins (Figure S4C), and overexpression of AMOT was not able to significantly enhance AL phosphorylation of LATS2-1041E that had a point mutation in the MOB binding domain (R657A, named MBD) (11) rendering it unable to bind MOB1 (Figure 5D). In addition, strong overexpression of MOB1A could stimulate similar levels of AL phosphorylation of LATS2-1041E in angiomotin deleted cells compared to wild-type cells (Figures 5E). Interestingly, despite having the same levels of LATS-AL phosphorylation the angiomotin deleted cells had a significant defect in YAP phosphorylation (Figure 5E), consistent with our earlier results suggesting a scaffolding role for angiomotins to promote LATS1/2 phosphorylation of YAP. Other evidence suggests that MOB1A and AMOT may have additive or synergistic effects on LATS1/2-AL phosphorylation. When both MOB1A and AMOT were moderately overexpressed (at levels where each alone barely enhanced LATS2-AL phosphorylation), we observed that co-expression of both proteins significantly increased AL phosphorylation of LATS2 (Figure 5F) beyond that observed for either protein alone. These results prompted us to test whether AMOT and MOB1A could promote AL phosphorylation even in the absence of HM phosphorylation (using LATS2-1041A). We found that although moderately expressed AMOT and MOB1A alone did not increase AL phosphorylation, expression of both proteins significantly increased AL phosphorylation of LATS2-1041A, albeit to lower levels than with wild-type LATS2 (Figure 5F). We think that the AL phosphorylation of LATS2-1041A was due to autophosphorylation and not phosphorylation by another kinase or endogenous LATS1/2 because a LATS2 kinase dead mutant did not show AL phosphorylation after MOB1A and AMOT expression (Figure 5F). Together, these

experiments indicate that AMOT and MOB1A can promote LATS2 autophosphorylation on the AL site independent of HM phosphorylation by upstream kinases.

Discussion

Previous studies showed that angiomotins promote LATS1/2 activity and YAP phosphorylation, but the mechanism was not known (21,25,32). We show here that angiomotins carry out these functions through multiple modes of action (Figure 6). Several lines of evidence are consistent with AMOT acting as a scaffolding protein. Previous studies have shown that angiomotins bind LATS1/2, NF2, and YAP (21,22,24-27,29,30), and here we show that they can also bind SAV1-MST1/2. Thus they have the ability to bring LATS1/2 together with both their activator and substrate. Interestingly, the SAV1 protein has also been proposed to have scaffolding function because it binds LATS1/2 (15) and MST1 (38-41). However, we found that cells lacking angiomotins show severe defects in binding between endogenous SAV1-MST1 and LATS1/2, suggesting that at physiological levels angiomotins are important for SAV/MST-LATS complex stability. This raises the question of how Hippo signaling complexes are assembled in animals like *Drosophila* that lack angiomotins. One possible explanation is a rearrangement in the PPxY motif ligands for the SAV WW domains. In *Drosophila* SAV binds to the LATS1/2 homolog WARTS through interaction between its WW domains and the 5 PPxY motifs in WARTS (14). Although similar mechanism could operate in humans, it may not be as robust because LATS1 and LATS2 contain fewer PPxY motifs than *Drosophila* WARTS (LATS1 and LATS2 have 2 and 1 PPxY motifs respectively). AMOT could act to recruit SAV1-MST1 to AMOT-LATS2 complexes via its 3 L/PPxY motifs (2 PPxY motifs and 1 LPxY motif), which could supply additional binding sites for the WW domains of SAV1. Thus *Drosophila* may not need angiomotins to recruit WW-domain proteins to WARTS because WARTS has 5 PPxY motifs instead of the two and one in LATS1 and LATS2 respectively. Utilization of the PPxY domains of AMOT to recruit WW-domain proteins to an AMOT-

LATS1/2 complex could provide another level of regulation, since we have previously shown that the L/PPxY motifs of AMOT are masked in the presence of F-actin. Here we showed that the phospho-mimetic point mutant (AMOT-175E) that disrupts F-actin binding by AMOT (22,26,33,34) (thereby making the L/PPxY motifs of AMOT available for binding to WW domains) is better than wild-type at promoting LATS2-HM phosphorylation. Together this would create a mechanism whereby reduction in F-actin frees AMOT to bind the WW domains of both SAV1 and YAP allowing it to promote LATS1/2 activation and YAP phosphorylation by bringing LATS1/2 together with its activator SAV1-MST1/2 and its target YAP.

This model may also be relevant to our observation showing weak co-immunoprecipitation between YAP and LATS2 except for when AMOT was co-expressed and reduced interaction between endogenous LATS1 and YAP in Amot-3KO cells. Here again, AMOT might be acting to provide additional L/PPxY sites for the WW domains of YAP to bind. Previous reports have given differing results regarding interaction between LATS2 and YAP, with some showing LATS2 interacts with YAP via the first WW domain of YAP (42-44), whereas another report failed to observe co-immunoprecipitation between YAP and LATS2 (45). These studies could be consistent with the interaction between YAP and LATS2 being weak, but observable if high enough levels of each protein are expressed. We previously showed that a competition for binding to AMOT between F-actin and YAP caused reduction in F-actin levels trigger AMOT to bind YAP and sequester it in the cytoplasm (22). Our current results suggest that the increased binding of AMOT to YAP caused by reduction in F-actin levels may help connect LATS1/2-AMOT complexes to YAP to promote its phosphorylation and cytoplasmic sequestration.

The other major discovery of this study was that AMOT can stimulate LATS2 to autophosphorylate on its activation loop. Previously MOB1 was the only protein known to enhance LATS1/2-AL phosphorylation (10,12). We observed that co-expression of

AMOT with MOB1A greatly enhanced LATS2-AL phosphorylation more than expression of either protein alone. Because both AMOT and MOB1A promote LATS1/2-HM site phosphorylation and this phosphorylation can trigger LATS1/2 to autophosphorylate at the AL site, we needed to be able to analyze LATS2-AL phosphorylation in isolation. To do this we used LATS2 mutants that had the HM phosphorylation site mutated to either non-phosphorylatable alanine or to phospho-mimetic glutamate. These results showed that activation or increased levels of AMOT and MOB1 can activate LATS2 even without activation by upstream kinases. The physiological relevance is at this point not clear. It is possible that AMOT and MOB1 always act in conjunction with LATS1/2-HM phosphorylation to promote activation of LATS1/2 through AL phosphorylation, or alternatively they might function under some circumstances to activate LATS2 independent of HM phosphorylation. Further studies will be required to address these possibilities.

Experimental Procedures

Cell culture and drug and siRNA treatments

HEK 293 were grown in Dulbecco's modified Eagle medium (DMEM, GIBCO) supplemented with 10% (v/v) fetal bovine serum (FBS, GIBCO) and 1% (v/v) penicillin/streptomycin (GIBCO) in a humidified incubator at 37°C with 5% CO₂. Cells were incubated with Latrunculin B (Lat B) for 45 minutes at concentrations of 1 μM (ENZO LIFE SCIENCES) or 0.2 μM (SIGMA). Okadaic acid (OA) was used at 100 μM for 2 hours. Angiomotins were stabilized with XAV-939 (Selleckchem) overnight at 10μM. Knockdowns with siRNA in Hela cells were performed using 75 nM of control (GL2) siRNA or 25 nM each of AMOT, AMOTL1, and AMOTL2 SMARTpool siRNA (Dharmacon M-015417, M-017595, and M-013232, respectively), and 18 ul of Lipofectamine RNAiMAX (Invitrogen) in 6 well plates following the manufacturer directions. After 48 hours cells were collected following Latrunculin B treatment as indicated.

Plasmids and mutagenesis

The term “well” below refers to a single well from a 12-well plate. AMOT (wt, S175E, and S175A (22)), AMOTL1, and AMOTL2 were expressed from pCDNA4-Myc-His at 400 ng per well for all assays except the experiments in Supplemental Figure S2A, where 750 ng were used. In Supplemental Figure S1B all three Angiomotins were expressed together from 500ng of plasmid per 10cm plate. SAV1 was expressed from pCDNA3-3HA, LATS2 (wt and phospho-mimetic LATS2-T1041E (21)) from pCDNA3.1-FLAG, MST1 from pCDNA-HA, all at 400 ng per well. MOB1A was expressed from pCDNA3.1-Myc at 400 ng per well and pEGFP at 200ng per well. AMOT-3PxY-Myc was described in (22). The LATS2-R657A MOB1 binding site mutant (MBD) was made on the 1041E background, using the QuickChange II Site mutagenesis kit (Agilent) and primers designed using the manufacturer’s web tool (<http://www.genomics.agilent.com/primerDesignProgram.jsp>). A similar procedure was followed to make SAV1-ww, but using QuickChange Lightning Multi (Agilent), and primers AACACAAATACAACCTCACGCGAGCCATG CTCTTGAGCGAGAAGGAC (for ww1), and CACAAATAAGAAGGCCCAAGCCAGGCAT GCCTGTGCTCCTAGTGTA (for ww2).

Antibodies

The following dilutions were used for antibodies in Western blots. The mouse anti- MST1 (Proteintech, 66663) was used at 1:2000. The mouse anti-tubulin (Proteintech, 66031), mouse anti-FLAG (Sigma, F3165), mouse anti-GFP (Santa Cruz, 9996), rabbit anti-Myc (Santa Cruz, 789), and mouse anti-Myc (Santa Cruz, 40) were used at 1:1000. The rabbit anti YAP (Proteintech, 13584), rabbit anti pYAP S127 (Cell Signaling, 4911), rabbit anti LATS1 (Cell Signaling, 3477), rabbit anti LATS2 (Cell Signaling, 5888), rabbit anti pLATS1-S909 (Cell Signaling, 9157), rabbit anti MST1/2 (Bethyl, A300-466A), and rabbit anti pLATS1-T1079 (Cell Signaling, 8654) were used at 1:500. The mouse anti-SAV1 (Santa Cruz, 374366) and mouse anti YAP (Santa Cruz,

101199) were used at 1:100. The rabbit anti-AMOT antibody was generated by Maria Fernandes (Université Laval, CHUQ-CHUL Research Centre) and used at 1:2000. The rabbit anti AMOTL1 was provided by Anthony Schmitt (The Pennsylvania State University) and used at 1:1000. The rabbit anti AMOTL2 was generated by Wenqi Wang (University of California Irvine) and used at 1:1000. Immunoprecipitation of endogenous proteins was carried out with rabbit anti-SAV1 (Cell Signaling, 13301) and rabbit anti LATS1 (Cell Signaling, 3477)

Cell transfection, immunoprecipitation, and Western blotting

For the experiment in Supplemental Figure S1B, AMOT, AMOTL1, and AMOTL2, were transfected at low levels into HEK293 Amot-3KO cells with FuGENE® HD (Promega), according to manufacturer’s protocol. Rescued A3KO cells were trypsinized 8 hours post transfection, re-plated, and incubated overnight. The next morning, cells were treated with Lat B as indicated. Lipofectamine 2000 (Invitrogen) was the transfection reagent of choice for all other experiments. Since the Hippo pathway gets activated by cell resuspension, all protein was collected from cells snap frozen with liquid nitrogen in the plates they were grown on. For experiments in Figures 3A, and 4A-B, a lysis buffer containing glycerol was used (10% glycerol, 20 uM Tris HCl pH 7, 137 mM NaCl, 2 mM EDTA, 1% NP40). The lysis buffer for all other experiments contained 1% NP-40, 150 mM NaCl, 2 mM EDTA, 6 mM Na₂HPO₄, and 4 mM NaH₂ PO₄. Both lysis buffers contained phosphatase and protease inhibitors (1mM PMSF, 1mM Na₃VO₄, and mammalian protease inhibitor cocktail (Sigma)) was added to frozen cells and a cells were solubilized with the help of a cell scraper and back and forth pipetting. Lysates were cleared by centrifugation at 15,000 g for 5 min at 4°C. Dynabeads (Invitrogen) were used for all immunoprecipitation experiments according to the manufacturer’s protocol. Non-specific IgG antibodies were typically used as a control for immunoprecipitation experiments. For experiments involving coimmunoprecipitation of proteins with different epitope tags, control experiments were done to

show that antibodies against the tag on the protein being immunoprecipitated did not bind to the co-expressed protein being tested for co-immunoprecipitation (Supplemental Figure S1G).

CRISPR mediated KO cell line development

Target sequences were selected using the web tool developed by the Zhang lab at M.I.T. (<http://crispr.mit.edu/>). Complementary oligos (below) containing the target sequence (uppercase) and appropriate overhangs (lowercase) were annealed and cloned into a variant of the px330 plasmid with puromycin resistance (46). AMOT:
caccGCCATACACCAGCAAGCCAC and
aaacGTGGCTTGCTGGTGTATGGC,
AMOTL1:
caccgCAAGTTCATGTTCTCGGTTG and
aaacCAACCGAGAACATGAACTTGc,
AMOTL2:
caccGCAGCGTGCGGTCTCAGTC and
aaacGACTGAGACGCGCACGCTGC, MST1:
caccGGATCGTTATGGAGTACTGT and
aaacACAGTACTCCATAACGATCC, MST2:
caccGTTATGGAGTACTGTGGCGC and
aaacGCGCCACAGTACTCCATAAC, SAV1:
caccGTTGGAATTGTTGGACCATGc and
aaacGCATGGTCCAACAATTCCAAC, NF2:
caccgGAACTCCATCTCGGCGTCCA and
aaacTGGACGCCGAGATGGAGTTC. KO cell lines were generated by transfecting 500 ng of the px330-based plasmid containing the CRISPR target sequence into HEK293 cells plated in 12-well plates using Lipofectamine 2000 (Invitrogen). The next day, cells were placed under selection for 48 hours with 1 µg/ml of Puromycin (GIBCO). Puromycin resistant cells were then heavily diluted and plated on 10 cm plates for colony isolation. Clonal lines were then expanded, and the expression of the target gene was determined by Western blot. Clonal lines that lacked expression of the target gene

were re-isolated again from single cells and tested by Western blot to ensure clonality. The sfGFP-MAP-AMOT expressing cells were created similarly to the KO cell lines except that instead of px330, we used MLM3636 (Addgene #43860) to generate the sgRNA (guide sequence acaccGAATTTCTCATCTCTATTGCg and aaaacGCAATAGAGATGAGAAATTc) that cuts near the start codon for AMOT), JDS246 (Addgene # 43861) to express Cas9, and a rescue plasmid containing sfGFP-MAP flanked by ~700 base pairs of genomic sequence immediately upstream and downstream of the AMOT start codon.

Pooled puromycin resistant cells were used for further analysis. Western blotting using AMOT and GFP antibodies was used to confirm that the cells expressed full length sfGFP-MAP-AMOT.

Quantification and Statistical Analysis

Students t-test analysis (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$) were performed with the Graphpad web tool (<https://www.graphpad.com/quickcalcs/ttest1.cfm>) and represent mean \pm SD for experiments done in triplicates. Western blot densitometry quantifications were either performed by LICOR Odyssey (when fluorescent probes were used), or by ImageJ (47) on images obtained with a Bio-Rad Chemi-Touch, when HRP conjugated antibodies were used.

Acknowledgments

This work was supported by NIH 5R01GM058406-18 grant to D.M. We thank Dr. Anthony Schmitt (The Pennsylvania State University) and Dr. Wenqi Wang (University of California Irvine) for antibodies against AMOTL1 and AMOTL2 respectively.

Conflict of Interest

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

References

1. Meng, Z., Moroishi, T., and Guan, K. L. (2016) Mechanisms of Hippo pathway regulation. *Genes Dev* **30**, 1-17
2. Zanconato, F., Cordenonsi, M., and Piccolo, S. (2016) YAP/TAZ at the Roots of Cancer. *Cancer Cell* **29**, 783-803

3. 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
4. Zhao, B., Li, L., Tumaneng, K., Wang, C. Y., and Guan, K. L. (2010) A coordinated phosphorylation by Lats and CK1 regulates YAP stability through SCF(beta-TRCP). *Genes Dev* **24**, 72-85
5. Liu, C. Y., Zha, Z. Y., Zhou, X., Zhang, H., Huang, W., Zhao, D., Li, T., Chan, S. W., Lim, C. J., Hong, W., Zhao, S., Xiong, Y., Lei, Q. Y., and Guan, K. L. (2010) The hippo tumor pathway promotes TAZ degradation by phosphorylating a phosphodegron and recruiting the SCF{beta}-TrCP E3 ligase. *J Biol Chem* **285**, 37159-37169
6. Chan, E. H., Nousiainen, M., Chalamalasetty, R. B., Schafer, A., Nigg, E. A., and Sillje, H. H. (2005) The Ste20-like kinase Mst2 activates the human large tumor suppressor kinase Lats1. *Oncogene* **24**, 2076-2086
7. Meng, Z., Moroiishi, T., Mottier-Pavie, V., Plouffe, S. W., Hansen, C. G., Hong, A. W., Park, H. W., Mo, J. S., Lu, W., Lu, S., Flores, F., Yu, F. X., Halder, G., and Guan, K. L. (2015) MAP4K family kinases act in parallel to MST1/2 to activate LATS1/2 in the Hippo pathway. *Nat Commun* **6**, 8357
8. Zheng, Y., Wang, W., Liu, B., Deng, H., Uster, E., and Pan, D. (2015) Identification of Happyhour/MAP4K as Alternative Hpo/Mst-like Kinases in the Hippo Kinase Cascade. *Dev Cell* **34**, 642-655
9. 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
10. Hergovich, A., Schmitz, D., and Hemmings, B. A. (2006) The human tumour suppressor LATS1 is activated by human MOB1 at the membrane. *Biochem Biophys Res Commun* **345**, 50-58
11. Hoa, L., Kulaberoglu, Y., Gundogdu, R., Cook, D., Mavis, M., Gomez, M., Gomez, V., and Hergovich, A. (2016) The characterisation of LATS2 kinase regulation in Hippo-YAP signalling. *Cell Signal* **28**, 488-497
12. Ni, L., Zheng, Y., Hara, M., Pan, D., and Luo, X. (2015) Structural basis for Mob1-dependent activation of the core Mst-Lats kinase cascade in Hippo signaling. *Genes Dev* **29**, 1416-1431
13. Yin, F., Yu, J., Zheng, Y., Chen, Q., Zhang, N., and Pan, D. (2013) Spatial organization of Hippo signaling at the plasma membrane mediated by the tumor suppressor Merlin/NF2. *Cell* **154**, 1342-1355
14. Tapon, N., Harvey, K. F., Bell, D. W., Wahrer, D. C., 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
15. Guo, C., Tommasi, S., Liu, L., Yee, J. K., Dammann, R., and Pfeifer, G. P. (2007) RASSF1A is part of a complex similar to the Drosophila Hippo/Salvador/Lats tumor-suppressor network. *Curr Biol* **17**, 700-705
16. Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S., Cordenonsi, M., Zanconato, F., Le Digabel, J., Forcato, M., Bicciato, S., Elvassore, N., and Piccolo, S. (2011) Role of YAP/TAZ in mechanotransduction. *Nature* **474**, 179-183
17. Fernandez, B. G., Gaspar, P., Bras-Pereira, C., Jezowska, B., Rebelo, S. R., and Janody, F. (2011) Actin-Capping Protein and the Hippo pathway regulate F-actin and tissue growth in Drosophila. *Development* **138**, 2337-2346
18. Sansores-Garcia, L., Bossuyt, W., Wada, K., Yonemura, S., Tao, C., Sasaki, H., and Halder, G. (2011) Modulating F-actin organization induces organ growth by affecting the Hippo pathway. *EMBO J* **30**, 2325-2335
19. Wada, K., Itoga, K., Okano, T., Yonemura, S., and Sasaki, H. (2011) Hippo pathway regulation by cell morphology and stress fibers. *Development* **138**, 3907-3914
20. Zhao, B., Li, L., Wang, L., Wang, C. Y., Yu, J., and Guan, K. L. (2012) Cell detachment activates the Hippo pathway via cytoskeleton reorganization to induce anoikis. *Genes Dev* **26**, 54-68

21. Paramasivam, M., Sarkeshik, A., Yates, J. R., 3rd, Fernandes, M. J., and McCollum, D. (2011) Angiotensin family proteins are novel activators of the LATS2 kinase tumor suppressor. *Mol Biol Cell* **22**, 3725-3733
22. Mana-Capelli, S., Paramasivam, M., Dutta, S., and McCollum, D. (2014) Angiotensins link F-actin architecture to Hippo pathway signaling. *Mol Biol Cell* **25**, 1676-1685
23. Feng, X., Degese, M. S., Iglesias-Bartolome, R., Vaque, J. P., Molinolo, A. A., Rodrigues, M., Zaidi, M. R., Ksander, B. R., Merlino, G., Sodhi, A., Chen, Q., and Gutkind, J. S. (2014) Hippo-independent activation of YAP by the GNAQ uveal melanoma oncogene through a trio-regulated rho GTPase signaling circuitry. *Cancer Cell* **25**, 831-845
24. Chan, S. W., Lim, C. J., Chong, Y. F., Pobbati, A. V., Huang, C., and Hong, W. (2011) Hippo Pathway-independent Restriction of TAZ and YAP by Angiotensin. *J Biol Chem* **286**, 7018-7026
25. Zhao, B., Li, L., Lu, Q., Wang, L. H., Liu, C. Y., Lei, Q., and Guan, K. L. (2011) Angiotensin is a novel Hippo pathway component that inhibits YAP oncoprotein. *Genes Dev* **25**, 51-63
26. Hirate, Y., Hirahara, S., Inoue, K., Suzuki, A., Alarcon, V. B., Akimoto, K., Hirai, T., Hara, T., Adachi, M., Chida, K., Ohno, S., Marikawa, Y., Nakao, K., Shimono, A., and Sasaki, H. (2013) Polarity-dependent distribution of angiotensin localizes hippo signaling in preimplantation embryos. *Curr Biol* **23**, 1181-1194
27. Leung, C. Y., and Zernicka-Goetz, M. (2013) Angiotensin prevents pluripotent lineage differentiation in mouse embryos via Hippo pathway-dependent and -independent mechanisms. *Nat Commun* **4**, 2251
28. Moleirinho, S., Hoxha, S., Mandati, V., Curtale, G., Troutman, S., Ehmer, U., and Kissil, J. L. (2017) Regulation of localization and function of the transcriptional co-activator YAP by angiotensin. *Elife* **6**
29. Yi, C., Troutman, S., Fera, D., Stemmer-Rachamimov, A., Avila, J. L., Christian, N., Persson, N. L., Shimono, A., Speicher, D. W., Marmorstein, R., Holmgren, L., and Kissil, J. L. (2011) A tight junction-associated Merlin-angiotensin complex mediates Merlin's regulation of mitogenic signaling and tumor suppressive functions. *Cancer Cell* **19**, 527-540
30. Li, Y., Zhou, H., Li, F., Chan, S. W., Lin, Z., Wei, Z., Yang, Z., Guo, F., Lim, C. J., Xing, W., Shen, Y., Hong, W., Long, J., and Zhang, M. (2015) Angiotensin binding-induced activation of Merlin/NF2 in the Hippo pathway. *Cell Res* **25**, 801-817
31. Couzens, A. L., Knight, J. D., Kean, M. J., Teo, G., Weiss, A., Dunham, W. H., Lin, Z. Y., Bagshaw, R. D., Sicheri, F., Pawson, T., Wrana, J. L., Choi, H., and Gingras, A. C. (2013) Protein interaction network of the mammalian Hippo pathway reveals mechanisms of kinase-phosphatase interactions. *Sci Signal* **6**, rs15
32. Wang, W., Huang, J., and Chen, J. (2011) Angiotensin-like proteins associate with and negatively regulate YAP1. *J Biol Chem* **286**, 4364-4370
33. Chan, S. W., Lim, C. J., Guo, F., Tan, I., Leung, T., and Hong, W. (2013) Actin-binding and cell proliferation activities of angiotensin family members are regulated by Hippo pathway-mediated phosphorylation. *J Biol Chem* **288**, 37296-37307
34. Dai, X., She, P., Chi, F., Feng, Y., Liu, H., Jin, D., Zhao, Y., Guo, X., Jiang, D., Guan, K. L., Zhong, T. P., and Zhao, B. (2013) Phosphorylation of angiotensin by Lats1/2 kinases inhibits F-actin binding, cell migration and angiogenesis. *J Biol Chem*
35. Adler, J. J., Johnson, D. E., Heller, B. L., Bringman, L. R., Ranahan, W. P., Conwell, M. D., Sun, Y., Hudmon, A., and Wells, C. D. (2013) Serum deprivation inhibits the transcriptional co-activator YAP and cell growth via phosphorylation of the 130-kDa isoform of Angiotensin by the LATS1/2 protein kinases. *Proc Natl Acad Sci USA*
36. Kim, M., Kim, M., Park, S. J., Lee, C., and Lim, D. S. (2016) Role of Angiotensin-like 2 mono-ubiquitination on YAP inhibition. *EMBO Rep* **17**, 64-78
37. Ma, H., McLean, J. R., Chao, L. F., Mana-Capelli, S., Paramasivam, M., Hagstrom, K. A., Gould, K. L., and McCollum, D. (2012) A highly efficient multifunctional tandem affinity purification approach applicable to diverse organisms. *Mol Cell Proteomics* **11**, 501-511

38. Callus, B. A., Verhagen, A. M., and Vaux, D. L. (2006) Association of mammalian sterile twenty kinases, Mst1 and Mst2, with hSalvador via C-terminal coiled-coil domains, leads to its stabilization and phosphorylation. *FEBS J* **273**, 4264-4276
39. 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
40. 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**
41. Hwang, E., Ryu, K. S., Paakkonen, K., Guntert, P., Cheong, H. K., Lim, D. S., Lee, J. O., Jeon, Y. H., and Cheong, C. (2007) Structural insight into dimeric 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
42. Kawahara, M., Hori, T., Chonabayashi, K., Oka, T., Sudol, M., and Uchiyama, T. (2008) Kpm/Lats2 is linked to chemosensitivity of leukemic cells through the stabilization of p73. *Blood* **112**, 3856-3866
43. Zhang, J., Smolen, G. A., and Haber, D. A. (2008) Negative regulation of YAP by LATS1 underscores evolutionary conservation of the Drosophila Hippo pathway. *Cancer Res* **68**, 2789-2794
44. Oka, T., Mazack, V., and Sudol, M. (2008) Mst2 and Lats kinases regulate apoptotic function of Yes kinase-associated protein (YAP). *J Biol Chem* **283**, 27534-27546
45. Jagannathan, R., Schimizzi, G. V., Zhang, K., Loza, A. J., Yabuta, N., Nojima, H., and Longmore, G. D. (2016) AJUBA LIM proteins limit Hippo activity in proliferating cells by sequestering the Hippo core kinase complex in the cytosol. *Mol Cell Biol*
46. Hainer, S. J., Gu, W., Carone, B. R., Landry, B. D., Rando, O. J., Mello, C. C., and Fazio, T. G. (2015) Suppression of pervasive noncoding transcription in embryonic stem cells by esBAF. *Genes Dev* **29**, 362-378
47. Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **9**, 671-675
48. Wang, W., Li, N., Li, X., Tran, M. K., Han, X., and Chen, J. (2015) Tankyrase Inhibitors Target YAP by Stabilizing Angiomotin Family Proteins. *Cell Rep* **13**, 524-532
49. Wang, H., Lu, B., Castillo, J., Zhang, Y., Yang, Z., McAllister, G., Lindeman, A., Reece-Hoyes, J., Tallarico, J., Russ, C., Hoffman, G., Xu, W., Schirle, M., and Cong, F. (2016) Tankyrase Inhibitor Sensitizes Lung Cancer Cells to Endothelial Growth Factor Receptor (EGFR) Inhibition via Stabilizing Angiomotins and Inhibiting YAP Signaling. *J Biol Chem* **291**, 15256-15266
50. Troilo, A., Benson, E. K., Esposito, D., Garib Singh, R. A., Reddy, E. P., Mungamuri, S. K., and Aaronson, S. A. (2016) Angiomotin stabilization by tankyrase inhibitors antagonizes constitutive TEAD-dependent transcription and proliferation of human tumor cells with Hippo pathway core component mutations. *Oncotarget* **7**, 28765-28782
51. Campbell, C. I., Samavarchi-Tehrani, P., Barrios-Rodiles, M., Datti, A., Gingras, A. C., and Wrana, J. L. (2016) The RNF146 and tankyrase pathway maintains the junctional Crumbs complex through regulation of angiomotin. *J Cell Sci* **129**, 3396-3411

Figure Legends

Figure 1. Angiomotins require MST1/2, SAV1, and NF2 to activate LATS1/2. A) HEK293 (wt) or HEK293 cells with MST1 and MST2 inactivated using CRISPR (MST1/2-KO) were transfected with a LATS2-FLAG expressing plasmid and either a control plasmid, or a plasmid for expressing Myc-tagged versions of each of the 3 angiomotin genes (AMOT, AMOTL1, AMOTL2). Cell lysates were analyzed by western blotting using antibodies against the LATS1/2-HM phosphorylation site (pLATS2-HM), Myc (Angiomotins-Myc), FLAG (LATS2-FLAG). Tubulin levels in cell lysates are also shown. B) HEK293 (wt) or HEK293 cells with AMOT, AMOTL1 and AMOTL2 inactivated using CRISPR (Amot-3KO)

were treated with or without Latrunculin B (Lat B) and cell lysates were analyzed by western blotting using antibodies against LATS1/2-HM phosphorylation, LATS1/2-AL phosphorylation, and LATS1. Tubulin levels in cell lysates are also shown. Quantification of LATS1/2-HM and LATS1/2-AL phosphorylation relative to untreated wt HEK293 cells are shown. (Mean \pm SD; n=3; NS= $P \geq 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, T-test). C) HEK293 (wt) or HEK293 cells with NF2 (NF2-KO) or SAV1 (SAV1-KO) inactivated using CRISPR were transfected with a control plasmid, or a plasmid for expressing Myc-tagged versions of AMOT, or the AMOT-S175E mutant. Cell lysates were analyzed by western blotting using antibodies against the LATS1/2-HM phosphorylation site (pLATS1/2-HM) and Myc (AMOT-Myc). Tubulin levels in cell lysates are also shown. D) HEK293 (wt) or MST1/2-KO HEK293 cells were transfected with LATS2-FLAG and AMOT-Myc expressing plasmids. Myc or control (IgG) antibodies were used for immunoprecipitations from cell lysates, and immune complexes and cell lysates were analyzed by western blotting for LATS2-FLAG and AMOT-Myc levels. Quantification of LATS2 levels in AMOT immune complexes is shown. (Mean \pm SD; n=3; *** $P \leq 0.001$, T-test). E) HEK293 (wt), NF2-KO, or SAV1-KO HEK293 cells were transfected with LATS2-FLAG and AMOT-Myc expressing plasmids. Myc or control (IgG) antibodies were used for immunoprecipitations from cell lysates, and immune complexes and cell lysates were analyzed by western blotting for LATS2-FLAG and AMOT-Myc levels. Quantification of LATS2 levels in AMOT immune complexes is shown. (Mean \pm SD; n=3; ** $P \leq 0.01$, NS= $P \geq 0.05$, T-test).

Figure 2. AMOT binds to active forms of LATS2. A) HEK293 cells were transfected with plasmids for expressing AMOT-Myc and either LATS2 or LATS2 with the AL and HM phosphorylation sites (S872 and T1041) mutated to alanine (LATS2-2A). Myc or control (IgG) antibodies were used for immunoprecipitations from cell lysates, and immune complexes and cell lysates were analyzed by western blotting for LATS2-FLAG and AMOT-Myc levels. Quantification of AMOT levels in LATS2 immune complexes is shown. (Mean \pm SD; n=3; ** $P \leq 0.01$, T-test). B) HEK293 cells were transfected with plasmids for expressing LATS2-FLAG and either AMOT-Myc, AMOT-175A-Myc, or AMOT-175E-Myc. Immunoprecipitations were performed on each cell lysate with either FLAG (LATS2-FLAG) or control (IgG) antibodies, and immune complexes and cell lysates were analyzed by western blotting for LATS2-FLAG and AMOT-Myc levels. Quantification of AMOT levels in LATS2 immune complexes is shown. (Mean \pm SD; n=3; ** $P \leq 0.01$, *** $P \leq 0.001$, T-test). C) HEK293 cells (wt) or MST1/2-KO cells were transfected with plasmids for expressing LATS2-FLAG and AMOT-175E-Myc. Immunoprecipitations were performed on each cell lysate with either FLAG (LATS2-FLAG) or control (IgG) antibodies, and immune complexes and cell lysates were analyzed by western blotting for LATS2-FLAG and AMOT-Myc levels. Quantification of AMOT levels in LATS2 immune complexes is shown. (Mean \pm SD; n=3; *** $P \leq 0.001$, T-test). D) HEK293 cells (wt) or MST1/2-KO cells were transfected with plasmids for expressing LATS2-T1041E-FLAG and AMOT-Myc. Immunoprecipitations were performed cell lysates with either Myc (AMOT-Myc) or control (IgG) antibodies, and immune complexes and cell lysates were analyzed by western blotting for LATS2-FLAG and AMOT-Myc levels. Quantification of LATS2 levels in AMOT immune complexes is shown. (Mean \pm SD; n=3; NS= $P \geq 0.05$, T-test)

Figure 3. AMOT promotes assembly of LATS2-SAV1-MST1 complexes. A) HEK293 cells expressing sfGFP-AMOT from the chromosomal locus were created using CRISPR mediated genome modification (see Experimental Procedures). Streptavidin beads were used to pull down (PD) sfGFP-MAP-AMOT protein (GFP-AMOT) (Wild-type HEK293 cells (wt) where the endogenous AMOT locus has not been tagged with the sfGFP-MAP tag were used as a control). Protein complexes on beads were analyzed by western blotting using antibodies against the indicated proteins. The levels of these proteins in cell lysates is shown. B) HEK293 cells were transfected with plasmids for expressing HA-MST1, HA-SAV1 and AMOT-Myc as indicated. Immunoprecipitations were performed cell lysates with either Myc (AMOT-Myc) or control (IgG) antibodies, and immune complexes and cell lysates were analyzed by western blotting for HA-MST1, HA-SAV1, and AMOT-Myc levels. C) HEK293 cells were transfected with plasmids for expressing HA-SAV1 or HA-SAV1 with both WW domains mutated (HA-SAV1-2ww) and

either AMOT-Myc or AMOT-3PxY-Myc (eliminates all 3 L/PPxY motifs in AMOT). Immunoprecipitations were performed on each cell lysate with either Myc (AMOT-Myc) or control (IgG) antibodies, and immune complexes and cell lysates were analyzed by western blotting for HA-SAV1 and AMOT-Myc levels. D) HEK293 cells were transfected with plasmids for expressing HA-SAV1, LATS2-FLAG, and AMOT-Myc as indicated. Immunoprecipitations were performed on each cell lysate with either FLAG (LATS2-FLAG) or control (IgG) antibodies, and immune complexes and cell lysates were analyzed by western blotting for LATS2-FLAG, HA-SAV1, and AMOT-Myc levels. Quantification of SAV/LATS2 ratios in LATS2 immune complexes for cells expressing LATS2 and SAV1 (LATS2+S) or LATS2, SAV1, and AMOT (LATS2+A+S) is shown. (Mean \pm SD; n=3; *P \leq 0.05, T-test). E) HEK293 cells were transfected with combinations of plasmids for expressing LATS2-FLAG, HA-MST1, HA-SAV1, and AMOT-Myc as indicated. Immunoprecipitations were performed on each cell lysate with either FLAG (LATS2-FLAG) or control (IgG) antibodies, and immune complexes and cell lysates were analyzed by western blotting for LATS2-FLAG, HA-MST1, HA-SAV1, and AMOT-Myc levels. Quantification of MST1/LATS2 and SAV1/LATS2 ratios in LATS2 immune complexes for cells co-expressing LATS2 and MST1 (L + M), LATS2, MST1 and SAV (L + M + S), LATS2, MST1 and AMOT (L + M + A), and LATS2, MST1, AMOT and SAV1 (L + M + A + S) is shown. (Mean \pm SD; n=3; *P \leq 0.05, **P \leq 0.01, ****P \leq 0.0001 T-test). F) HEK293 cells were transfected with combinations of plasmids for expressing GFP-LATS2, FLAG-YAP2, and AMOT-Myc as indicated. Immunoprecipitations were performed on each cell lysate with either GFP (LATS2-GFP) or control (IgG) antibodies, and immune complexes and cell lysates were analyzed by western blotting for LATS2-GFP, FLAG-YAP2, and AMOT-Myc levels.

Figure 4. Angiomotins function as scaffolds for the Hippo pathway in vivo. A) HEK293 (wt) and Amot-3KO HEK293 cells were grown to high density and serum starved for 4 hours. Immunoprecipitations were performed on cell lysates with either anti-LATS1 or control (IgG) antibodies, and immune complexes and cell lysates were analyzed by western blotting for AMOT, SAV1, LATS1, and YAP levels. (LATS1 bg: residual LATS1 signal after stripping). B) HEK293 (wt) and Amot-3KO HEK293 cells were grown as in (A). Immunoprecipitations were performed on cell lysates with either anti-SAV1 or control (IgG) antibodies, and immune complexes and cell lysates were analyzed by western blotting for AMOT, MST1, SAV1, LATS1, and YAP levels.

Figure 5. AMOT promotes LATS2-AL activation loop phosphorylation independent of LATS2-HM phosphorylation. A) HEK293 (wt) or Amot-3KO HEK293 cells were transfected with a control plasmid, or a plasmid for expressing HA-MST1. Cell lysates were analyzed by western blotting using antibodies against the LATS1/2-HM phosphorylation site (pLATS1/2-HM), HA (HA-MST1), and LATS1. Tubulin levels in cell lysates are also shown. Quantification of LATS1/2-HM phosphorylation is shown. (Mean \pm SD; n=3; NS=P \geq 0.05, *P \leq 0.05, **P \leq 0.01, ****P \leq 0.0001, T-test). B) HEK293 (wt) or Amot-3KO HEK293 cells were transfected with a control plasmid, or a combination of plasmids (L + M + S) for expressing LATS2-FLAG, HA-MST1, and HA-SAV1. Cell lysates were analyzed by western blotting using antibodies for LATS2-HM and LATS2-AL phosphorylation, FLAG (LATS2-FLAG), and HA (HA-MST1 and HA-SAV1). Tubulin levels in cell lysates are also shown. Quantification of LATS1/2-HM phosphorylation is shown. (Mean \pm SD; n=3; NS=P \geq 0.05, **P \leq 0.001, T-test). C) HEK293 cells were transfected with a control plasmid, LATS2-1041E-FLAG, AMOT-175E-Myc as indicated. Cell lysates were analyzed by western blotting using antibodies for LATS2-AL phosphorylation (pLATS2-AL), FLAG (LATS2-1041E-FLAG), and Myc (AMOT-175E-Myc). Tubulin levels in cell lysates are also shown. Quantification of LATS1/2-AL phosphorylation relative to LATS2-1041E is shown. L-1041E: LATS2-1041E, A-175E: AMOT-175E. (Mean \pm SD; n=3; ****P \leq 0.0001, T-test). D) HEK293 cells were transfected with a control plasmid, or LATS2-1041E-FLAG, LATS2-1041E-MBD-FLAG (MBD is a LATS2 mutant defective for binding MOB1), AMOT-175E-Myc as indicated. Cell lysates were analyzed by western blotting using antibodies for LATS2-AL phosphorylation (pLATS2-AL), FLAG (LATS2-1041E-FLAG or LATS2-1041E-MBD-FLAG), and Myc (AMOT-Myc). Tubulin levels in cell lysates are

also shown. Quantification of LATS1/2-AL phosphorylation relative to LATS2-1041E-FLAG levels is shown. L-1041E: LATS2-1041E, A: AMOT-175E. (Mean \pm SD; n=3; NS= $P \geq 0.05$, ** $P \leq 0.01$, T-test). E) HEK293 (wt) or Amot-3KO HEK293 cells were transfected with a plasmid for expressing LATS2-1041E-FLAG and either a control plasmid or a plasmid for expressing Myc-MOB1A. Cell lysates were analyzed by western blotting using antibodies for LATS2-AL phosphorylation (pLATS2-AL), YAP phosphorylation on S127 (pYAP-S127), FLAG (LATS2-1041E-FLAG), and Myc (Myc-MOB1A). Tubulin levels in cell lysates are also shown. Quantification of LATS1/2-AL and pYAP-S127 phosphorylation is shown. (Mean \pm SD; n=3; NS= $P \geq 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, T-test). F) HEK293 cells were transfected with a control plasmid, LATS2-FLAG, LATS2-1041E-FLAG, kinase dead LATS2 (LATS2-KD-FLAG), AMOT-Myc, or Myc-MOB1A as indicated. Cell lysates were analyzed by western blotting using antibodies for LATS2-AL phosphorylation (pLATS2-AL), FLAG (LATS2-FLAG), Myc (AMOT-Myc and Myc-MOB1A). Tubulin levels in cell lysates are also shown. L: LATS2, L-1041A: LATS2-1041A, L-KD: LATS2-kinase dead, A: AMOT-175E, M: MOB1A. Quantification of LATS1/2-AL phosphorylation is shown. (Mean \pm SD; n=3; NS= $P \geq 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, **** $P \leq 0.0001$, T-test).

Figure 6. Model for AMOT function in Hippo signaling. (A) When F-actin levels are high AMOT is bound to F-actin and poorly interacts with its binding partners. (B) If F-actin levels are reduced, AMOT becomes phosphorylated by LATS and is free to bind YAP, SAV-MST, and LATS. Assembly of this complex allows MST to phosphorylate LATS on the HM site. Both AMOT phosphorylation and LATS HM phosphorylation enhance AMOT-LATS binding. (C) To become fully active LATS must autophosphorylate on the AL site. HM phosphorylation enhances LATS autophosphorylation. In addition, we show that both MOB1 and AMOT enhance LATS-AL phosphorylation independent of any effects they have on HM phosphorylation. (D) The ability of fully active LATS to phosphorylate YAP may be enhanced by formation of a LATS-AMOT-YAP complex. Note that the model is speculative and its purpose is illustrate the primary steps involved in AMOT activation of Hippo signaling. The Hippo pathway is regulated at many other levels, which could be occurring at the same time but are not shown for simplicity. It is not presently clear whether complexes exist in cells as pictured with all components simultaneously bound to each other.

Figure 1

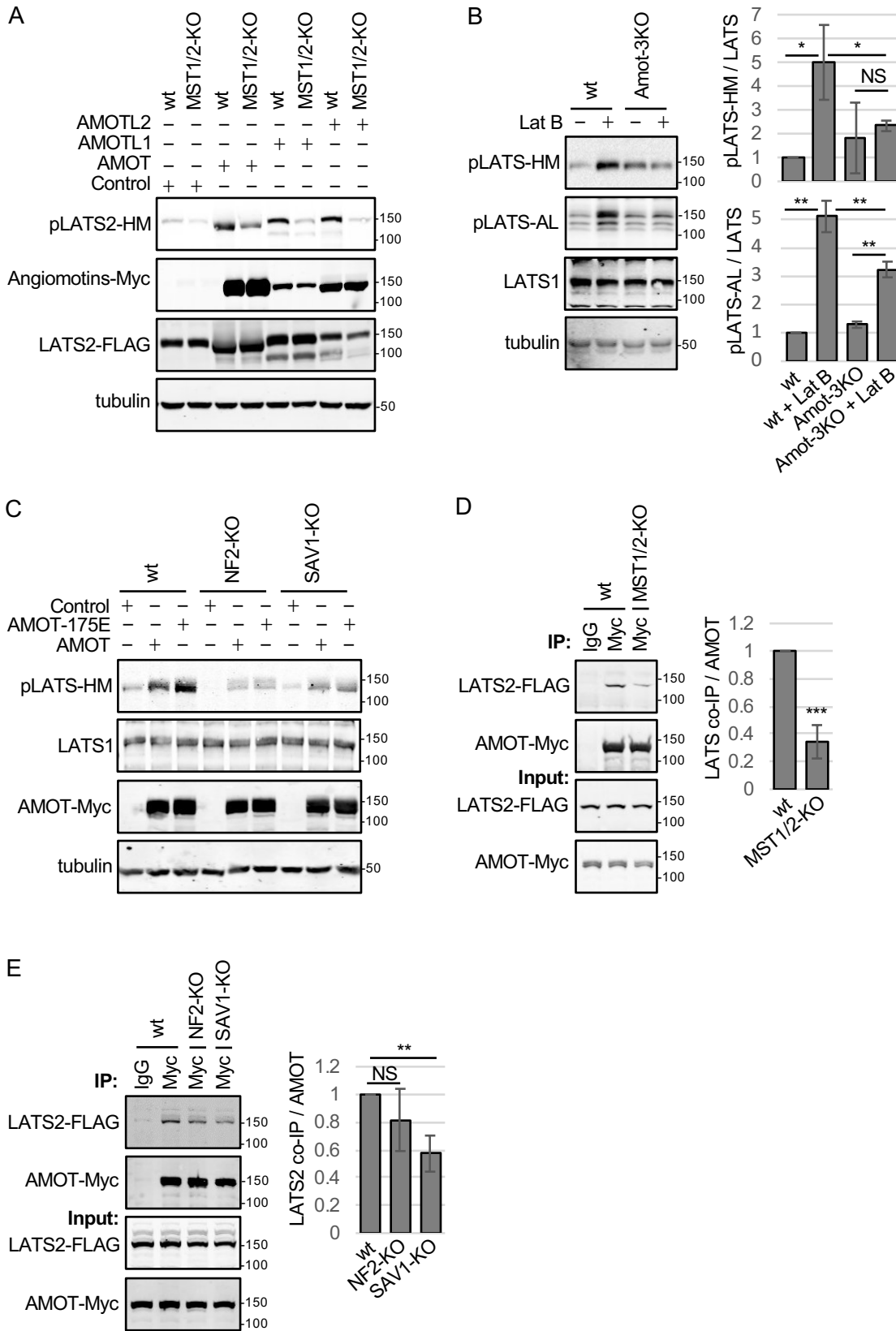


Figure 2

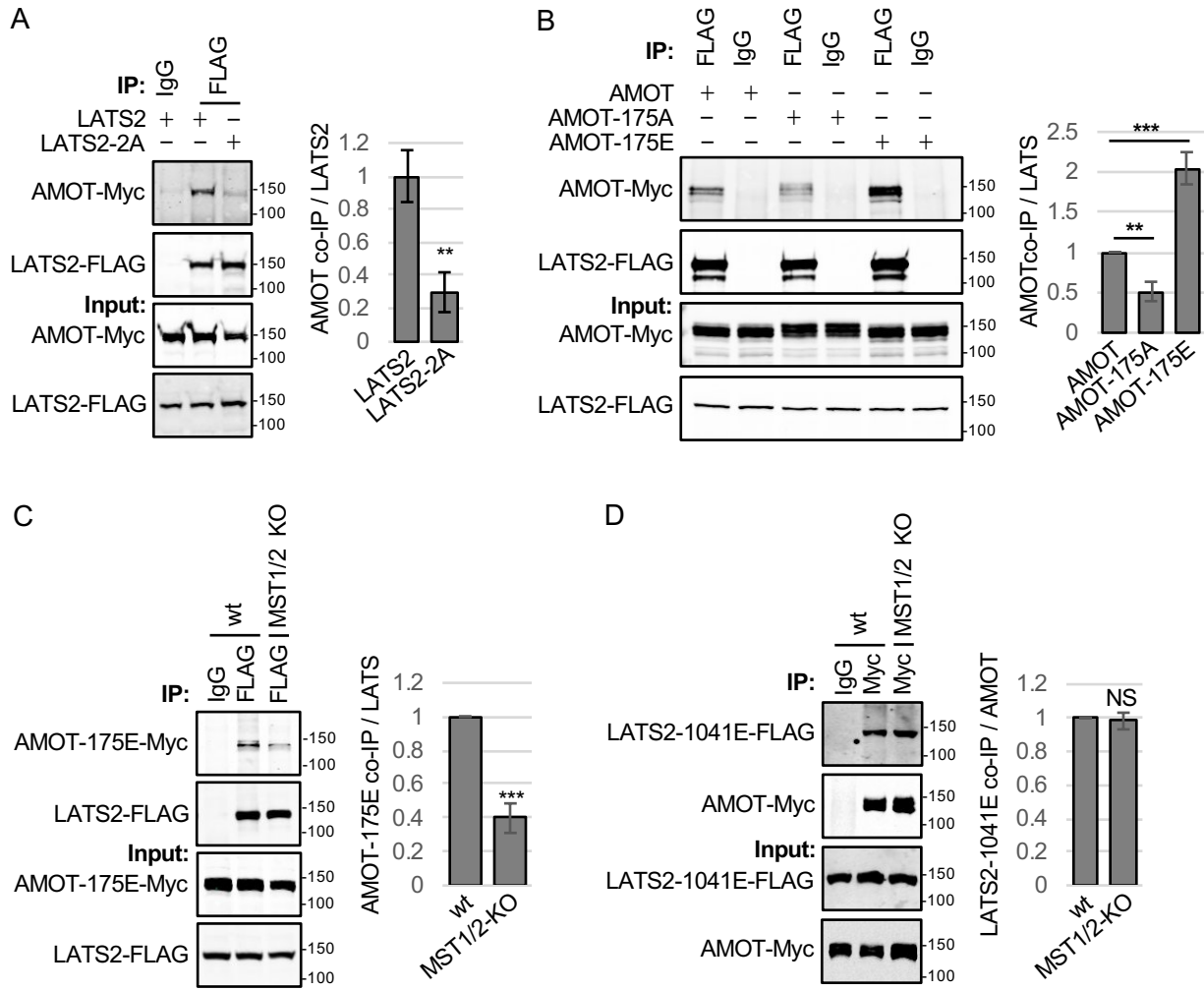


Figure 3

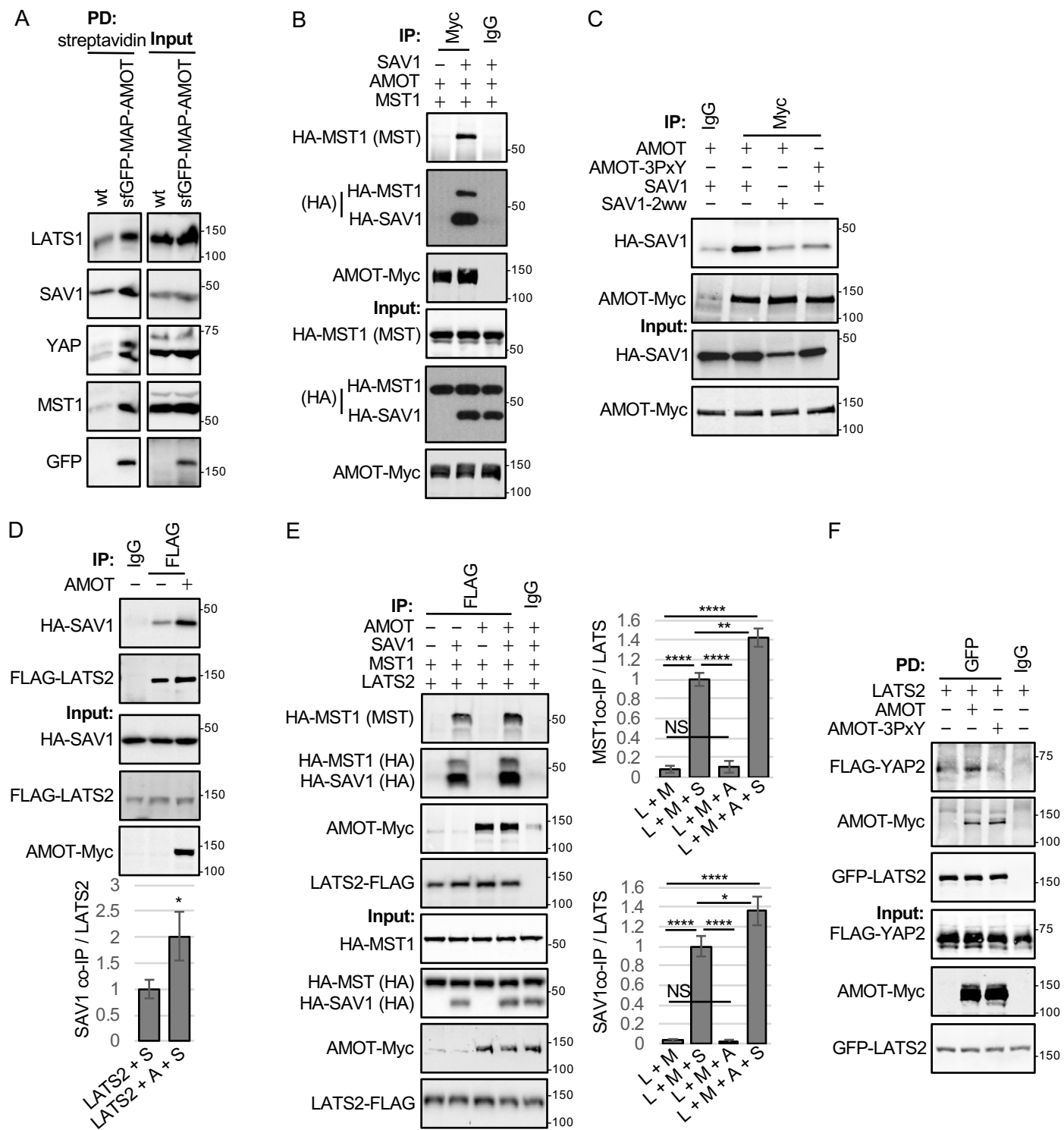


Figure 4

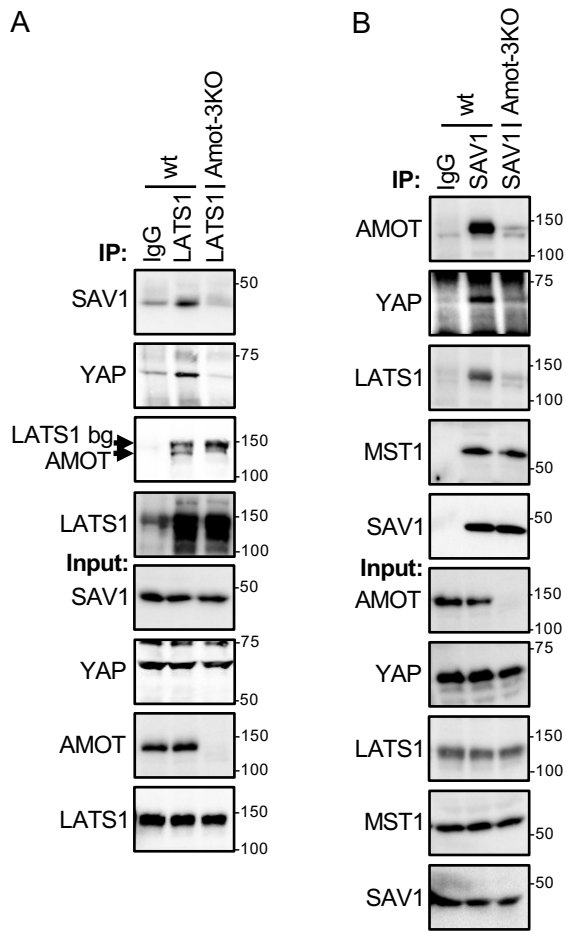


Figure 5

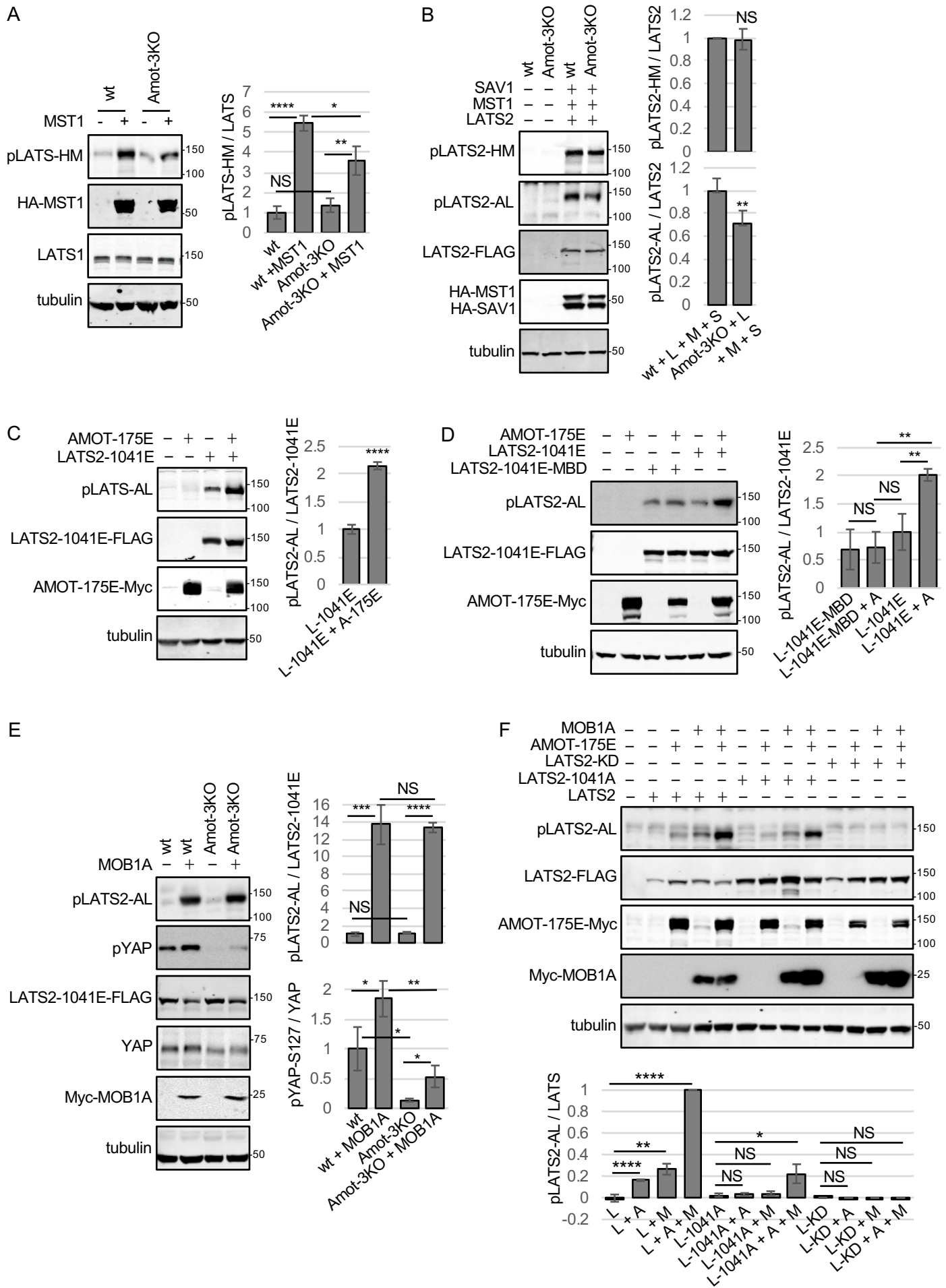
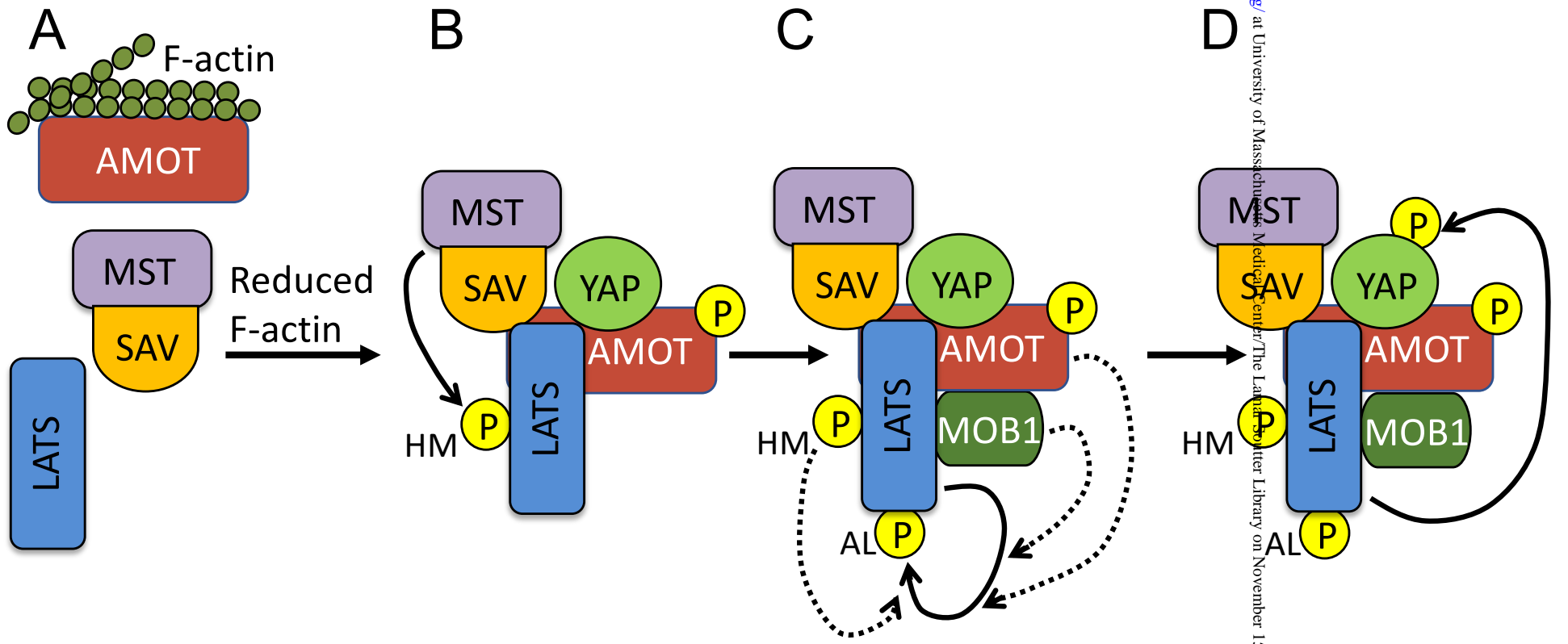


Figure 6



Angiotensins stimulate LATS kinase autophosphorylation and act as scaffolds that promote Hippo signaling

Sebastian Mana-Capelli and Dannel McCollum

J. Biol. Chem. published online September 28, 2018

Access the most updated version of this article at doi: [10.1074/jbc.RA118.004187](https://doi.org/10.1074/jbc.RA118.004187)

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