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Angiomotins link F-actin architecture to Hippo pathway signaling

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ABSTRACT The Hippo pathway regulates the transcriptional coactivator YAP to control cell proliferation, organ size, and stem cell maintenance. Multiple factors, such as substrate stiffness, cell density, and G protein-coupled receptor signaling, regulate YAP through their effects on the F-actin cytoskeleton, although the mechanism is not known. Here we show that angiomotin proteins (AMOT130, AMOTL1, and AMOTL2) connect F-actin architecture to YAP regulation. First, we show that angiomotins are required to relocalize YAP to the cytoplasm in response to various manipulations that perturb the actin cytoskeleton. Second, angiomotins associate with F-actin through a conserved F-actin-binding domain, and mutants defective for F-actin binding show enhanced ability to retain YAP in the cytoplasm. Third, F-actin and YAP compete for binding to AMOT130, explaining how F-actin inhibits AMOT130-mediated cytoplasmic retention of YAP. Further, we find that LATS can synergize with F-actin perturbations by phosphorylating free AMOT130 to keep it from associating with F-actin. Together these results uncover a mechanism for how F-actin levels modulate YAP localization, allowing cells to make developmental and proliferative decisions based on diverse inputs that regulate actin architecture.

INTRODUCTION

The Hippo pathway regulates contact inhibition of cell growth, cell proliferation, apoptosis, stem cell maintenance and differentiation, and the development of cancer in mammals and flies (Yu and Guan, 2013). The core Hippo pathway in mammals consists of the MST1/2 kinases, which activate the LATS1/2 kinases, which in turn phosphorylate and inhibit the homologous transcriptional coactivators YAP and TAZ (hereafter referred to as YAP), causing them to relocalize from the nucleus to the cytoplasm. Nuclear YAP promotes growth, proliferation, and stem cell maintenance. YAP localizes to the nucleus in cells at low density, and at high density YAP exits the nucleus and cells stop proliferation. How YAP is regulated in response to cell density is not known, although recent evidence suggests that the organization of the actin cytoskeleton contributes through an unknown mechanism (Dupont et al., 2011; Fernandez et al., 2011; Sansores-Garcia et al., 2011; Wada et al., 2011; Zhao et al., 2012). In addition, G protein-coupled receptors have been shown to modulate Hippo signaling through F-actin (Molina et al., 2012; Yu et al., 2012). F-actin can influence YAP activity through both Hippo pathway-dependent (Wada et al., 2011; Zhao et al., 2012; Kim et al., 2013) and Hippo pathway-independent mechanisms (Dupont et al., 2011; Amgona et al., 2013). Intriguingly, angiomotin family members AMOT, AMOTL1, and AMOTL2 can also inhibit YAP both in a Hippo pathway-independent manner by binding and sequestering YAP in the cytoplasm and by activating the YAP inhibitory kinase LATS (Hirota et al., 2012; Mora et al., 2012; Yu et al., 2012). F-actin can influence YAP activity through both Hippo pathway-dependent (Wada et al., 2011; Zhao et al., 2012; Kim et al., 2013) and Hippo pathway-independent mechanisms (Dupont et al., 2011; Amgona et al., 2013). Given the ability to associate with actin structures (Ernkvist et al., 2008; Gagne et al., 2009), we hypothesized that angiomotins might mediate the effects of F-actin on YAP. Here we report evidence in support of this hypothesis.
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**RESULTS**

The N-terminal Hippo pathway regulatory domain of angiomotins contains an actin-binding motif. Overexpression of the long form of AMOT (AMOT130) causes formation of large F-actin bundles that also contain AMOT130–AB, AMOT130 with a deletion in the actin-binding region (AMOT130-AB), or a fragment containing the actin-binding region fused to GFP (AMOT130(157-191)) and is aged at low densities. Cells were stained for AMOT130 using antimyc or GFP antibodies and for F-actin using phalloidin. DNA was stained with DAPI. Bar, 20 µm. (A) U2OS cells were transfected with a plasmid for expression of full-length myc-tagged AMOT130 and then stained for AMOT130 (using antimyc antibodies) and endogenous myosin IIA, which is a marker for stress fibers. Bar, 20 µm. (B) U2OS cells were transfected with a plasmid for expression of full-length myc-tagged AMOT130, amino acids 100–200 of AMOT130 (AMOT130(100–200)), AMOT130 with a deletion in the actin-binding region (AMOT130-AB), or a fragment containing the actin-binding region fused to GFP (AMOT130(157–191)) and stained at low densities. Cells were stained for AMOT130 using antimyc or GFP antibodies and for F-actin using phalloidin. DNA was stained with DAPI. Bar, 20 µm. (C) Representation of angiomotin protein features, including the actin-binding region flanked by YAP-binding motifs. (D) Alignment of the amino-terminal region of human AMOT130, AMOTL1, and AMOTL2 is shown. The region containing the actin-binding region (underlined) and LATS phosphorylation site are indicated (boxed). Numbers correspond to amino acid numbers for AMOT130.

**FIGURE 1:** AMOT130 associates with F-actin through a domain in its N-terminus. (A) U2OS cells were transfected with plasmids for expression of myc-tagged full-length AMOT130, amino acids 100–200 of AMOT130 and AMOT130(100–200), AMOT130 with a deletion in the actin-binding region (AMOT130-AB), or a fragment containing the actin-binding region fused to GFP (AMOT130(157–191)) and stained at low densities. Cells were stained for AMOT130 using antimyc or GFP antibodies and for F-actin using phalloidin. DNA was stained with DAPI. Bar, 20 µm. (B) U2OS cells were transfected with a plasmid for expression of full-length myc-tagged AMOT130, amino acids 100–200 of AMOT130 and then stained for AMOT130 (using antimyc antibodies) and endogenous myosin IIA, which is a marker for stress fibers. Bar, 20 µm. (C) Representation of angiomotin protein features, including the actin-binding region flanked by YAP-binding motifs. (D) Alignment of the amino-terminal region of human AMOT130, AMOTL1, and AMOTL2 is shown. The region containing the actin-binding region (underlined) and LATS phosphorylation site are indicated (boxed). Numbers correspond to amino acid numbers for AMOT130.

Actin binding of AMOT130 is regulated by LATS2 kinase. Of interest, the conserved sequence block in the actin-binding region of angiomotins contains a perfect consensus LATS phosphorylation site (HXRXXS; serine 175 in AMOT130; Figure 1, C and D), suggesting that LATS might regulate the actin-binding properties of angiomotins. Consistent with this idea, expression of LATS2, but not kinase-dead LATS2, could disrupt both AMOT130 localization to actin fibers and its actin-bundling activity (Figure 2, A–C). Mutation of the putative LATS phosphorylation site in the actin-binding region of AMOT130 or AMOTL2 blocked in vitro phosphorylation of each protein by LATS2 (Supplemental Figure S2A) and blocked the ability of LATS2 to inhibit the actin-bundling and localization activity of AMOT130 (Figure 2, A–C). In contrast, AMOT130-S175E could not localize or bundle actin (Figure 2, A–C). Thus LATS2...
FIGURE 2: LATS2 inhibits association of AMOT130 with F-actin. (A) U2OS cells were transfected with the indicated AMOT130 and LATS2 plasmids and imaged at low densities. Cells were stained for AMOT130 (Myc), F-actin using phalloidin, and LATS2 or LATS2-KD (FLAG). DNA was stained with DAPI. Bar, 20 µm. (B, C) Quantification of the phenotypes of the cells in A. Graphs represent the average from three experiments (n ≥ 100 each), and error bars indicate SD of the averages. Brackets on top of bars represent statistical significance (Fisher test, p < 0.00001). (D) Immunostaining of endogenous AMOT130, phospho-AMOT130, and actin. HEK 293T cells were stained with phalloidin to visualize actin and with the indicated antibodies. (E) HEK 293T cells growing at increasing densities were costained with anti-AMOT130 and anti-phospho-AMOT130 (p-AMOT130). DNA was stained with DAPI. Bar, 20 µm.
phosphorylation of AMOT130 inhibits its localization to F-actin. Localization of endogenous AMOT130 in 293T cells supported this conclusion. In cells at low density, AMOT130 was observed to co-localize with actin fibers (Figure 2D). In contrast, phospho-AMOT130 (analyzed with phospho-serine 175-specific antibodies; Hlate et al., 2013) did not co-localize with F-actin fibers and was instead observed at regions of cell-cell contact (Figure 2D). As cells became more dense and established more cell-cell contacts, increased phospho-AMOT130 staining was observed at cell-cell junctions (Figure 2B). Endogenous phospho-AMOT130 was only occasionally seen at vesicles, like the phospho-mimic AMOT130-S175E mutant (see Discussion).

Because the LATS phosphorylation site is in the middle of the AMOT130 actin-binding region, we hypothesized that just as phosphorylation inhibits AMOT130 actin binding, binding of AMOT130 to F-actin might interfere with phosphorylation by LATS. To test this model in vivo, we first determined whether AMOT130 could bind directly to F-actin in vivo. Consistent with in vitro data, recombinant AMOT130 (Figures 3A and Supplemental Figure S2B), but not AMOT130-S175E (Figure 3A), could bind to F-actin, whereas both AMOT130 and AMOT130-S175E bound recombinant YAP (Figure 3B). Using in vitro kinase assays, we observed that LATS2 could phosphorylate AMOT130 in the absence but not in the presence of F-actin (Figure 3C). This result is consistent with recent observations showing that LATS phosphorylation of AMOT130 in vivo is enhanced by disruption of F-actin (Dai et al., 2013). Thus, LATS may act, after perturbations that reduce F-actin levels, to phosphorylate free AMOT130 to keep it from rebinding to F-actin.

Actin binding-deficient mutants of AMOT130 show enhanced YAP inhibition

Previous studies showing that YAP is inhibited by F-actin disruption could be explained if an inhibitor of YAP was kept sequestered by binding to F-actin. AMOT130 functions in this manner; then mutants that cannot bind F-actin should have enhanced ability to inhibit YAP in vivo. Therefore we tested whether localization to F-actin affected the ability of AMOT130 to inhibit YAP nuclear localization and transcriptional activity. Wild-type and mutant forms of AMOT130 were transfected into U2OS cells, and the localization of endogenous YAP was examined (Figure 4, A–C). In control cells to AMOT130 YAP was mainly in the nucleus. Wild-type AMOT130 and AMOT130-S175A were able to cause limited translocation of YAP to the cytoplasm (only in cells with high AMOT130 expression levels; Figure 4C). Of interest, the AMOT130-S175A mutant was less effective than wild-type AMOT130 at bringing YAP to the cytoplasm. In contrast, the mutants that could not bind F-actin AMOT130-AB or AMOT130-S175E were much more effective at shifting YAP to the cytoplasm (Figure 4A, similar to when AMOT130 was coexpressed with AMOT130 on vesicles Figure 4A), similar to when AMOT130 was coexpressed with AMOT130 on vesicles (Figure 4A). Together these results show that F-actin binding antagonizes the ability of AMOT130 to inhibit YAP nuclear localization and function.

F-actin and YAP compete for binding to AMOT130

Binding to F-actin could inhibit the ability of AMOT130 to direct YAP to the cytoplasm by blocking either AMOT130 activation of LATS or binding of AMOT130 to YAP. To address this question, we made AMOT130 mutants that were specifically defective at either activating LATS2 or binding YAP. To disrupt interaction between AMOT130 and YAP, we mutated the three L/PPXY motifs in AMOT130 that are known to mediate interaction between AMOT130 and the W W domain of YAP (Chan et al., 2011; Wang et al., 2011; Zhao et al., 2011; Agkret et al., 2013). Because AMOT130 mutants defective at activating LATS2 had not been identified, we mutated blocks of conserved residues in the amino terminus of AMOT130, which was known to be required for LATS2 activation (Passek et al., 2011), and tested their ability to promote IAT52 phosphorylation of YAP. Because mutation of residues 13–27 abolished the ability of AMOT130 to activate LATS2 (Figure 4F), this domain was termed the IAT52 activation domain (IAD). Of interest, both AMOT130-AB and wild-type AMOT130 promoted IAT52 phosphorylation of YAP to a similar degree, suggesting that F-actin binding might not regulate AMOT130 activation of IAT52. Next we used these mutants to test how F-actin regulates the ability of AMOT130 to promote cytoplasmic localization of YAP. Expression of different versions of AMOT130-AB with deletions in or adjacent to the L/PPXY motifs, with the IAD domain only (no contribution Figure 4B). This suggests that F-actin binding primarily interferes with AMOT130 binding to YAP.

Because the F-actin-binding domain of AMOT130 is closely flanked by YAP-binding motifs (Figure 1C), we hypothesized that F-actin and YAP might compete for binding to AMOT130, which...
FIGURE 4: Actin and YAP compete for binding to AMOT130, and AMOT130 mutants that cannot bind F-actin are more efficient at inhibiting YAP. A, B) U2OS cells were transfected with either control plasmid or one of the indicated AMOT130 plasmids. The next day, cells were stained for endogenous YAP and scored for the percent of cells with more YAP in the nucleus than the cytoplasm (N > C), more in the cytoplasm than the nucleus (C > N), or equal signal in the cytoplasm and nucleus (C = N). A) Example images. B) Average from three experiments (n = 100 each), and the error bars indicate SD of the averages. Brackets on top of bars represent statistical significance (Fisher test, *p < 0.00001, **p < 0.02). Bar, 20 µm. C) The AMOT130, AMOT130-S175A, AMOT130-S175E, and AMOT130-ΔAB expression levels...
could allow F-actin levels to modulate the ability of AMOT130 to bind to YAP. Consistent with this idea, overexpression of YAP in U2O5 cells blocked localization of coexpressed AMOT130 to F-actin, and both proteins localized to vesicles (Supplemental Figure S3D). When next tested, both showed whether F-actin and YAP compete for binding to AMOT130. AMOT130 (on beads) was allowed to bind F-actin and then incubated in the presence or absence of increasing anounts of YAP (Figure 4G). We observed that high YAP concentrations displaced F-actin from AMOT130, showing that YAP and actin compete for binding to AMOT130. Together, these data point toward competition between F-actin and YAP for binding to AMOT130, which could explain how actin modulates AMOT130 regulation of YAP.

Angiomotins mediate the effects of actin perturbation on YAP localization

Various treatments that perturb F-actin (Supplemental Figure S4A) cause YAP to exit the nucleus (Dupont et al., 2011; Fenemad et al., 2011; Sanson et al., 2011; Wada et al., 2011; Zhao et al., 2012). Experiments include 1) F-actin depolymerization by latrunculin B or cytochalasin D; 2) serum withdrawal, which acts through G protein-coupled receptors to affect the actin cytoskeleton (Maple et al., 2012; Mo et al., 2012; Yu et al., 2012); 3) type 2 myosin inhibition, which affects actin-stabilizing proteins (Dupont et al., 2011); and 4) increased cell density (Dupont et al., 2011). We found that angiomotins and LATS are required for regulation of YAP localization in each case. We used small interfering RNA (siRNA) to knockdown AMOT130, AMOT1, and AMOTL2 in HEK293A and MCF10A cells (Supplemental Figure S4B). Although knockdown of individual angiomotins had limited effects, knockdown of all three caused nuclear retention of YAP and maintenance of YAP activity after F-actin depolymerization, type 2 myosin inhibition, serum withdrawal, and increased cell density in HEK293A and MCF10A cells (Figures 5, A–D, and Supplemental Figure 5C–F). Note that the effect of triple knockdown in HEK293A cells after latrunculin B treatment or serum starvation could be caused by overexpression of AMOT130 or AMOT1, or both. Figures 5, A and B, show knockdowns of both AMOT130 and AMOT1, triple angiomotin knockdown blocked cytoplasmic accumulation of YAP to a similar degree as LATS1/2 knockdown after latrunculin B treatment but had a significantly stronger effect than LATS1/2 knockdown alone (Figure 5A). This fits with studies suggesting that F-actin structures that respond to mechanical forces such as stress fibers are involved in YAP regulation (Dupont et al., 2011; Wada et al., 2011). Although we show that AMOT130 and AMOT1 can bind F-actin in vivo, it will be important in future studies to determine whether AMOT130 can distinguish between types of F-actin structures in vivo. A direct competition between F-actin and YAP appears to underlie the ability of F-actin to keep AMOT130 from binding and sequestering YAP in the cytoplasm. Angiomotin proteins are major effectors of the effects of F-actin on YAP, since they are required for the cytoplasmic retention of YAP that occurs when F-actin is disrupted. Together, these results suggest a model (Figure 5E) in which AMOT130 accumulates on F-actin structures and stimulates the release of these structures, such as increased cell density, resulting in release of AMOT130, allowing it to bind and inhibit YAP.

In single cells, we quantified and correlated with endogenous YAP localization. The graphs plot the average AMOT130 levels for individual cells (plotted based on AMOT130 levels) and are scored for those with more YAP in the nucleus than cytoplasm (N > C, solid symbols) or not (N < C, open symbols). D) Endogenous YAP and phospho-AMOT130 (p-AMOT130) staining in HEK193T cells with or without treatment with latrunculin B for 15 min. DNA is stained with DAPI. Bar, 20 μm. E) U2O5 cells were transfected with the same AMOT130 plasmid as in A, as well as an 8XGluT1–luciferase YAP-dependent promotor plasmid and a plasmid with the SV40 promoter driving Renilla luciferase. The next day, cells were scored for luciferase activity and firefly and Renilla luciferase activity in each sample. The levels of luciferase were normalized to the level of Renilla luciferase in each sample. Error bars indicate the SD of the averages. F) LATS2, YAP, and the induced AMOT130 plasmid were transfected into HEK293 cells, and the levels of AMOT130, LATS2, YAP, and phospho-YAP were analyzed by Western blotting. The experiment was done in triplicate, and error bars indicate the SD of the averages. G) Competition between actin and YAP for binding to AMOT130. Recombinant MBP-AMOT130 protein on beads was prebound to F-actin then incubated in the presence or absence of increasing amounts of recombinant GST–VAP2. The levels of bound proteins and input are shown.
FIGURE 5: Angiomotins and LATS are required to efficiently inhibit YAP after F-actin disturbance. (A) HEK293A cells were transfected with control siRNA (luciferase) or siRNA against AMOT130, AMOTL1, AMOTL2, a combination of all three angiomotins (triple KD), or a combination of LATS1 and LATS2 (LATS1+2), as indicated. To test for off-target effects, plasmids for expressing either AMOT130 (RAMOT130) or AMOTL2 (RAMOTL2) were transfected the next day to test for rescue of the triple-knockdown phenotype. Forty-eight hours later, cells were treated with either latrunculin B (see example images) or blebbistatin (Blebb) and then fixed and stained for localization of endogenous YAP. Cells were scored for the percentage of cells with more YAP in the nucleus than the cytoplasm (N>C), more in the cytoplasm than the nucleus (C>N), or equal signal in the cytoplasm and nucleus (C=N). Brackets on top of bars represent statistical significance (Fisher test, p < 0.0005). (B) HEK293A cells were manipulated as in A, except that instead of drug treatment, cells were shifted to media without serum for 2 h and then fixed and stained for endogenous YAP localization (% of cells).
F-actin–binding domain, causes accumulation of large endosomal-like compartments (Heber et al., 2010). In future studies it will be important to determine whether localization of AMOT130–YAP complexes to vesicles and the plasma membrane plays a role in YAP regulation.

There has been some question about the importance of IATS for F-actin–dependent regulation of YAP (Dupont et al., 2011; Yu et al., 2012; Zhao et al., 2012; Amonogna et al., 2013). Our work, together with other studies, suggests that IATS functions together with angiomotins to regulate YAP in response to F-actin perturbation. We show that IATS contributes to cytoplasmic retention of YAP after F-actin disruption and seem withdrawal, and several reports have shown that IATS becomes activated and inhibits YAP by direct phosphorylation when F-actin is disrupted (Wada et al., 2011; Zhao et al., 2012; Amonogna et al., 2013). Our work indicates that activated IATS can also act through angiomotins to inhibit YAP. IATS phosphorylation of AMOT130 is enhanced by F-actin disruption in vitro (Dai et al., 2013), and we show that the ability of IATS2 to phosphorylate AMOT130 in vitro is increased in the absence of F-actin. From this study, as well as from several recent reports, it is clear that IATS phosphorylation of AMOT130 inhibits its ability to bind F-actin (Adler et al., 2013b; Chan et al., 2013; Dai et al., 2013; Hata et al., 2013). We show that IATS phosphorylation blocks AMOT130 binding to F-actin, allowing it to bind YAP and sequester it in the cytoplasm. IATS phosphorylation of AMOT130 appears to have additional functions. A recent study indicates that AMOT130 phosphorylation could also enhance AMOT130 binding to the WD domain-containing E3 ubiquitin ligase AIP4, which can both stabilize AMOT130 and promote YAP degradation (Aderet al., 2013a,b). It seems as if to be determined whether AIP4, like YAP, directly competes with F-actin for binding to AMOT130. Recent studies also suggest that AMOT130 phosphorylation by IATS could enhance the AMOT130–IATS interaction (Hata et al., 2013) and have effects on the actin cytoskeleton (Dai et al., 2013). Thus IATS can promote cytoplasmic localization of YAP in response to F-actin depolymerization by phosphorylating AMOT130 in addition to its well-characterized function in phosphorylating YAP (Figure 5B).

The competition between F-actin and YAP for binding to AMOT130 could also provide a F-actin-independent mechanism for F-actin-dependent regulation of YAP. The F-actin-dependent mechanism could allow for combinatorial regulation of YAP activity based on both inputs that affect the actin cytoskeleton, such as cell density, and inputs that affect IATS activity, such as cell-cell contacts (Kim et al., 2011), as was recently suggested (Amonogna et al., 2013). Together this work shows that F-actin, angiomotins, and IATS form a regulatory module that controls YAP in response to diverse inputs such as changes in cell density, substrate stiffness, and G protein-coupled receptor signaling (Halber et al., 2012).

Materials and Methods

Cell lines

Human HEK 293, HEK293A, Hela, and U2OS cell lines were grown in DMEM (Gibco, Grand Island, NY) supplemented with 10% (vol/vol) fetal bovine serum (Gibco), 1% (vol/vol) penicillin/streptomycin (Invitrogen, Grand Island, NY), and 1% (vol/vol) 2% (vol/vol) ECGS (Gibco). Human mammary epithelial MCF10A cells were cultured in MEGM BulletKit (Lonza, Hopkinton, MA) with all additives except for the gentamicin phosphate (Sigma-Aldrich, St. Louis, MO) and 1% penicillin/streptomycin (Invitrogen). All cell lines were cultured in a humidified incubator at 37°C with 5% CO₂.

In vitro kinase assays and luciferase assays

For detection of IATS2–mediated phosphorylation of angiomotins with P–32, HEK 293 cells were transfected in 12-well plates with IATS2, various angiomotin constructs, and IATS activator MST1 (Sav, and M0B1), using Lipofectamine 2000 (Invitrogen). Forty hours after transfection, cells were lysed in 1 mm unphosphorylated buffer 60 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet-P-40, 2% glyceral supplemented with 1 protease inhibitor cocktail (Sigma-Aldrich), 100 mM sodium vanadate (Sigma-Aldrich), and 50 mM sodium fluoride (Sigma-Aldrich), and lysates were cleared by centrifugation at 13,000 rpm for 10 min at 4°C. Protein lysate (000 µg) was processed for in vitro phosphorylation as described previously (Pannaschnik et al., 2011). Both IATS2 and angiomotin proteins were immunoprecipitated together on the same beads. Kinase assays and Western blotting were carried out as previously described (Pannaschnik et al., 2011).

For kinase assays in the presence of F-actin, IATS2–FLAG was transfected into HEK293 cells together with its activators, MST1 and M0B1. After 24 h, IATS2 was purified in phosphate buffer using anti-FLAG M2 antibody (Sigma-Aldrich) and magnetic protein G beads (Sigma-Aldrich) following the manufacturer’s directions. MAb–binding protein MBP–AMOT130 was expressed and purified as described and eluted with 20 mM maltose in supplemented actin buffer 5 mM Tris·Cl, pH 8.0, 0.2 mM CaCl₂, 50 mM KC1, 2 mM MgCl₂, and 1 mM ATP/Cytoskeleton, Denver, CO) for 30 min at 4°C. Eluted AMOT130 (10 µl, 0.5 µg) was then incubated with or without 10 µl of F-actin (see pribip description, 5 mM final concentration) for 15 min at room temperature. Control reactions were taken to 20 µl with supplemented actin buffer. For kinase reactions the AMOT130/F-actin mix was added to IATS2–bound beads prewashed with supplemented actin buffer. After incubation at 30°C for 30 min, kinase reactions were stopped by boiling in SDS sample buffer. Samples were then subjected to SDS–PAGE, and phospho-AMOT130 was detected by Western blotting using a phosphospecific antibody.

YAP. Cells were scored as h A. Example p in images ages shown. Brackets on top of bars represent statistical significance. *p < 0.0005, **p < 0.005. C) Lentiviral infection was used to induce either control shRNA (directed against luciferase) or shRNA against all three angiomotins (AMOT130, AMOTL1, and AMOTL2; triple knockdown) into MC10A cells. Sixty hours after infection, cells were left untreated, treated with cholera toxin (Cytoskeleton), or treated with 20 mM maltose in supplemented actin buffer. After incubation at 30°C for 30 min, kinase reactions were stopped by boiling in SDS sample buffer. Samples were then subjected to SDS–PAGE, and phospho-AMOT130 was detected by Western blotting using a phosphospecific antibody.
Luciferase assays were performed in U2OS and HeLa cells 24 h after transfection. All transfections were performed in 12-well plates using Lipofectamine 2000 and a concentration of 300 ng of TIE2-luc (94615; Addgene, Cambridge, MA), 20 ng of pRL-SV40P (referred to as renilla, 27163; Addgene), and the described AMOT130 plasmid 500 ng for U2OS and 25 ng for HeLa cells. Cells were treated and reactions performed following directions described in the Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

Cell starvation and drug treatments
HEK293A cells were starved for 2 h in DMEM without serum. MCF10A cells were starved overnight in DMEM/F12 supplemented with 100 ng/mL cholera toxin (Sigma-Aldrich) and 1% penicillin and streptomycin (Invitrogen). Latrunculin B and cytochalasin D were used at 1 μM each, except for the phospho-AMOT130/YAP staining (Figure 4D), for which cells were incubated for only 15 min. Note that cytochalasin D was used to disrupt F-actin in MCF10A cells because latrunculin B was too toxic in these cells. Blebbistatin was used at 25 μM for 1 h.

Immunocytochemistry
U2OS, HeLa, and MCF10A cells cultured on coverslips were fixed in phosphate-buffered saline PBS/0.1% formaldehyde for 10 min and permeabilized with 0.1% Triton X-100 and 5% nonfat dry milk for 30 min. Cells were subsequently incubated with appropriate primary antibodies for 1–2 h at room temperature. They were washed three times in PBS with 0.1% Triton X-100 and incubated with Alexa Fluor-conjugated secondary antibodies (referred to as renilla, 27163; Addgene), and the described antibody solution when appropriate. After these washes, coverslips were mounted on slides using Vectashield (Vector Laboratories, Burlingame, CA) and viewed using fluorescent microscopy (Nikon Eclipse E600). Images were acquired using a cooled charge-coupled device camera (ORCA-ER; Hamamatsu, Bridgewater, NJ). Image processing and analysis were carried out with IPLab Spectrum software (Signal Analytics, Vienna, VA) and ImageJ software (Schneider et al., 2012).

In vivo protein-binding assays
AMOT130 and AMOT130-S175E were cloned in pDEST-MBP provided by Maoz W. Alatsoumis lab using Gateway (Invitrogen) standard procedures. M BP-AMOT130 and M BP-AMOT130-S175E were expressed with 1 mM isopropyl-β-d-thiogalactoside (IPTG) for 4 h at 25°C and shaking. M BP fusion proteins were purified with maltose beads HEB (Invitrogen, MA) in phosphate buffer 60 mM NaH2PO4, 150 mM NaCl, 10 mM 3-mercaptoethanol, 0.1% Triton, and 1 mM phenylmethylsulfonyl fluoride (PMSF) following the manufacturer’s directions. Expression of glutathione-S-transferase (GST)-YAP2 (pEG-EX52;vector;GE Healthcare, Piscataway, NJ) was induced by addition of 1 mM IPTG for 2 h at 25°C, and then GST-YAP2 was purified with glutathione beads (GE Healthcare) in phosphate buffer and eluted with 20 mM glutathione for 30 min. Normal mouse ascites was purchased as part of the Actin Binding Protein Kit (Cytoskeleton) and was polymerized for 1 h at 25°C following the manufacturer’s directions. For the in vivo pulldown experiments, beads-bound AMOT130 and AMOT130-S175E were incubated for 30 min at room temperature with eluted GST-YAP2 and/or 5 μM F-actin in phosphate buffer containing 2 mM ATP and 2 mM MgCl2 to keep F-actin stable. Actin Binding Protein kit manual. Com petition assays were assembled as follows. First, a constant amount of actin was incubated with M BP-AMOT130 beads for 15 min at room temperature. Then a constant volume of either GST elution buffer or increasing amounts of elution GST-YAP2 were added as indicated in Figure 3F. Samples were then incubated for an additional 30 min. All samples were washed once with phosphate buffer and boiled in SDS-PAGE sample buffer for the cosedimentation experiments, M BP-AMOT130 was eluted from maltose beads with 10 mM maltose for 30 min and incubated with actin as for 30 min at room temperature. Samples were then centrifuged at 150,000 g in a Beckman TLX bench-top ultracentrifuge for 1.5 h. Pellets were suspended in the same volume as the supernatant and boiled in SDS–PAGE loading buffer. Protein samples were subjected to SDS-PAGE and Western blotting with the specified antibodies.

Plasmids
Sources for plasmids used in this study were described previously (Paaz et al., 2011). A LAMOT130, AMOT1L1, and AMOTL2 constructs were expressed from pcDNA4-Myc-His. Large deletion mutants in AMOT130, AMOT1L1, and AMOTL2 were constructed using PCR followed by subcloning. Point and small deletion mutations in AMOT130 and AMOTL2 were made using the Quick-Change II Site-Directed Mutagenesis kit (Stratagene, Santa Clara, CA). All localization studies were performed in a 12-well format. At the various time points, plasmids were transfected at 600 ng/well, and LATS2 constructs pCDNA 3.1-LATS2-FLAG and pCDNA3.1-LATS2-KD-FLAG were transfected at 400 ng/well.

Antibodies
Mou se anti-tubulin and mouse antiFLAG (M2) were purchased from Sigma-Aldrich. The rabbit anti-YAP (sc15407), mouse anti-YAP (sc10199), rabbit anti-Myc (sc789), mouse anti-Myc 9E10 (sc67), mouse anti-S6 (sc9202), mouse anti-AMOT130 4B-4 (sc166924), and goat anti-AMOT1L1 (B-2501) were from Santa Cruz Biotechnology (Dallas, TX). M yosin IIb was purchased from Cell Signaling Technology (9403; Beverly, MA). The rabbit antiAM OT antibody was generated by the Fernandes lab (CHUQ-CHUL Research Center, University of Laval, Quebec City, Canada). Rabbit antiAM OT was provided by Anthony Schmitt (Pennsylvania State University, State College, PA). AMOT130 and AMOTL2 were cloned in pDEST15 using Gateway (Invitrogen) and were expressed with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 4 h at 25°C and shaking.

Plasmid transfection
Cells were transfected using Lipofectamine 2000 LipoMAX LipoLique (Invitrogen). Cells were cultured for 48 h after transfection. The only exceptions were experiments with cells at high densities, for which siRNAs were transfected twice at 40 nM (second transfection after 24 h), and cells were fixed after 72 h of the last transfection. For rescuing experiments, plasmids were transiently transfected as above. To generate a triple knockdown, stable AMOT130, AMOT1L1, and AMOTL2 were transfected at 400 ng/well, and LATS2 was transfected at 600 ng/well.

siRNA/shRNA transfection
Knockdowns in HEK293A cells were performed using 30 nM control siRNA or SMARTpools-RNA (Dharmacon, Lafayette, CO) and 3 μl of RNAMAX Lipoarcin in (Invitrogen). Cells were cultured for 48 h after transfection. The only exceptions were experiments with cells at high densities, for which siRNAs were transfected twice at 40 nM (second transfection after 24 h), and cells were fixed after 72 h of the last transfection. For rescuing experiments, plasmids were transiently transfected as above. To generate a triple knockdown, stable AMOT130, AMOT1L1, and AMOTL2 were transfected at 400 ng/well, and LATS2 was transfected at 600 ng/well.

In vivo protein-binding assays
AMOT130 and AMOT130-S175E were cloned in pDEST-MBP provided by Maoz W. Alatsoumis lab using Gateway (Invitrogen) standard procedures. MBP-AMOT130 and MBP-AMOT130-S175E were expressed with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 4 h at 25°C and shaking. MBP fusion proteins were purified with maltose beads HEB (Invitrogen, MA) in phosphate buffer 60 mM NaH2PO4, 150 mM NaCl, 10 mM 3-mercaptoethanol, 0.1% Triton, and 1 mM phenylmethylsulfonyl fluoride (PMSF) following the manufacturer’s directions. Expression of glutathione-S-transferase (GST)-YAP2 (pEG-EX52; vector; GE Healthcare, Piscataway, NJ) was induced by addition of 1 mM IPTG for 2 h at 25°C, and then GST-YAP2 was purified with glutathione beads (GE Healthcare) in phosphate buffer and eluted with 20 mM glutathione for 30 min. Normal mouse ascites was purchased as part of the Actin Binding Protein Kit (Cytoskeleton) and was polymerized for 1 h at 25°C following the manufacturer’s directions. For the in vivo pulldown experiments, beads-bound AMOT130 and AMOT130-S175E were incubated for 30 min at room temperature with eluted GST-YAP2 and/or 5 μM F-actin in phosphate buffer containing 2 mM ATP and 2 mM MgCl2 to keep F-actin stable. Actin Binding Protein kit manual. Competition assays were assembled as follows. First, a constant amount of actin was incubated with MBP-AMOT130 beads for 15 min at room temperature. Then a constant volume of either GST elution buffer or increasing amounts of elution GST-YAP2 were added as indicated in Figure 3F. Samples were then incubated for an additional 30 min. All samples were washed once with phosphate buffer and boiled in SDS-PAGE sample buffer for the cosedimentation experiments, MBP-AMOT130 was eluted from maltose beads with 10 mM maltose for 30 min and incubated with actin as for 30 min at room temperature. Samples were then centrifuged at 150,000 g in a Beckman TLX bench-top ultracentrifuge for 1.5 h. Pellets were suspended in the same volume as the supernatant and boiled in SDS–PAGE loading buffer. Protein samples were subjected to SDS-PAGE and Western blotting with the specified antibodies.
knockdown cells were infected with a combination of AMOTL130 and AMOT1 lentiviral supernatants. At the same time, stable control cells were infected with control siloumamptan. At the control.

Viral supernatants were generated by the shRNA Core Facility, Wilmington, MA). Target mRNA levels were measured relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using KAPA SYBR Fast-Master Mix Universal kit (Kapa Biosystems, Waltham, MA). Real-time quantitative PCR was performed using KAPA SYBR Fast-Master Mix Universal kit (Kapa Biosystems, Waltham, MA). Target mRNA levels were measured relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. The following primers were used. GAPDH-F, CTCCTGCACCAC-CAGGAAAACATGAGAAACAAATTGG (AMOT130) or TGGTGGAA-TATCTCATCTA (AMOTL130).

Real-time quantitative PCR

After appropriate treatments to cells on 6-well (MCF10A) or 12-well plates (HEK293A), media was aspirated off and cells were lysed with TRIzol Life Technologies, Grand Island, NY) and processed for total RNA isolation according to the manufacturer's protocol. cDNA was prepared by oligo-dT (Promega) using SuperScript II Reverse Transcriptase (Invitrogen). Real-time quantitative PCR was performed using KAPA SYBR Fast-Master Mix Universal kit (Kapa Biosystems, Waltham, MA). Target mRNA levels were measured relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. The following primers were used. GAPDH-F, CTCCTGCACCAC-CAGGAAAACATGAGAAACAAATTGG (AMOT130) or TGGTGGAA-TATCTCATCTA (AMOTL130).

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