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. Furthermore, 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 modulates YAP localization, allowing cells to make development 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 (Miller et al., 2012; Mo et al., 2012; Yu et al., 2012). F-actin can influence YAP activity through both Hippo pathway (ATS)-dependent (Wada et al., 2011; Zhao et al., 2012; Kim et al., 2013) and Hippo pathway–independent mechanisms (Dupont et al., 2011; Amagana et al., 2013). Intriguingly, angiomotins (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 (Hippo dependent; Chan et al., 2011; Paramasivam et al., 2011; Wang et al., 2011; Zhao et al., 2011; Hirate et al., 2013; Leung and Zernicka-Goetz, 2013). Given their 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.
localize to vesicular structures [see Discussion], as observed for AMOT80 [Heller et al., 2010], a shorter form of AMOT lacking the actin-binding region.) In addition, a small fragment (AMOT130 residues 157–191) centered around the residues deleted in AMOT130-AB localized to F-actin structures when fused to green fluorescent protein (GFP; Figure 1A).

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 to or bundle actin (Figure 2, A–C). Thus LATS2 phosphorylation site.
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 AM OT130 inhibits its localization to F-actin. Localization of endogenous AM OT130 in 293T cells supported this conclusion. In cells at low density, AM OT130 was observed to co-localize with F-actin fibers (Figure 2D). In contrast, phospho-AM OT130 (analyzed with phospho-serine 175-specific antibodies; Hiatt et al., 2013) did not co-localize with F-actin fibers and instead observed at regions of cell–cell contact (Figure 2D). As cells became more dense and established more cell-cell contacts, increased phospho-AM OT130 staining was observed at cell-cell junctions (Figure 2B). Endogenous phospho-AM OT130 was only occasionally seen at vesicles, like the phospho-mimetic AM OT130-S175E mutant (see Discussion).

Because the LATS phosphorylation site is in the middle of the AM OT130 actin-binding region, we hypothesized that just as phosphorylation inhibits AM OT130 actin binding, binding of AM OT130 to F-actin might interfere with phosphorylation by LATS. To test this model in vivo, we first determined whether AM OT130 could bind directly to F-actin in vivo. Consistent with in vitro data, recombinant AM OT130 (Figures 3A and Supplemental Figure S2B), but not AM OT130-S175E (Figure 3A), could bind to F-actin, whereas both AM OT130 and AM OT130-S175E bound recombinant YAP (Figure 3B). Using in vivo kinase assays, we observed that LATS2 could phosphorylate AM OT130 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 AM OT130 in vivo is enhanced by disruption of F-actin (Daliet al., 2013). Thus LATS may act, after perturbations that reduce F-actin levels, to phosphorylate free AM OT130 to keep it from rebinding to F-actin.

Actin binding–deficient mutants of AM OT130 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. If AM OT130 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 AM OT130 to inhibit YAP nuclear localization and transcriptional activity. Wild-type and mutant forms of AM OT130 were transfected into U2OS cells, and the localization of endogenous YAP was examined (Figure 4, A–C). In control cells to AM OT130, YAP remained primarily in the nucleus. Wild-type AM OT130 and AM OT130-S175A were able to cause limited translocation of YAP to the cytoplasm (only in cells with high AM OT130 expression levels; Figure 4C). Of interest, the AM OT130-S175A mutant was less effective than wild-type AM OT130 at bringing YAP to the cytoplasm. In contrast, the mutants that could not bind F-actin (AM OT130-AB or AM OT130-S175E) were much more effective at shifting YAP to the cytoplasm (Figure 4A, similar to when AM OT130 was co-expressed with AM OT130 on vesicles Figure 4A). Similarly, soon after disruption of F-actin in 293T cells using latrunculin B, endogenous YAP was observed to co-localize with S175-phosphorylated endogenous AM OT130 on structures possibly vesicles) near the plasma membrane (Figure 4D).

When we assayed transcription from a synthetic YAP-dependent promoter Dupont et al., 2011), although all forms of AM OT130 are expressed similarly (Supplemental Figure S3B) and show inhibition of YAP (probably due to overexpression), we again found that the AM OT130 mutants that could not bind F-actin were much more effective at inhibiting YAP (Figure 4E and Supplemental Figure S3C). Together these results show that F-actin binding antagonizes the ability of AM OT130 to inhibit YAP nuclear localization and function.

F-actin and YAP compete for binding to AM OT130

Binding to F-actin could inhibit the ability of AM OT130 to direct YAP to the cytoplasm by blocking either AM OT130 activation of LATS or binding of AM OT130 to YAP. To address this question, we made AM OT130 mutants that were specifically defective at activating LATS2 or binding YAP. To disrupt interaction between AM OT130 and YAP, we mutated the three L/PPXY motifs in AM OT130 that are known to mediate interaction between AM OT130 and the WW domain of YAP (Chan et al., 2011; Wang et al., 2011; Zhao et al., 2011; Adler et al., 2013a). Because AM OT130 mutants defective at activating LATS had not been identified, we mutated blocks of conserved residues in the amoeba homolog of AM OT130, which was known to be required for LATS2 activation (Passa et al., 2011, and tested their ability to promote LATS2 phosphorylation of YAP. Because mutation of residues 13–27 abolished the ability of AM OT130 to activate LATS2 (Figure 4F), this domain was termed the LATS activation domain (LAD). Of interest, both AM OT130-AB and wild-type AM OT130 promoted LATS2 phosphorylation of YAP to a similar degree, suggesting that F-actin binding might not regulate AM OT130 activation of LATS2. Next we used these mutants to test how F-actin regulates the ability of AM OT130 to promote one cysteine in localization of YAP. Expression of different versions of AM OT130-AB with deletions in either the YAP-binding motif or the LAD demonstrated that the enhanced ability of AM OT130-AB to translocate YAP to the cytoplasm depended on motif, the L/PPXY motif, with the LAD mediating only a minor contribution (Figure 4B). This suggests that F-actin binding primarily interacts with AM OT130 binding to YAP.

Because the F-actin-binding domain of AM OT130 is closely flanked by YAP-binding motifs (Figure 1C), we hypothesized that F-actin and YAP might compete for binding to AM OT130, which

**Figure 3:** LATS phosphorylation of AM OT130 prevents its association with F-actin, and AM OT130 binding to F-actin inhibits LATS phosphorylation. A, B) In vitro binding assays between recombinant MBP-AM OT130 or MBP-AM OT130-S175E and purified F-actin. A) MBP-AM OT130 protein bound to beads was used to pull down PD-F-actin or GST-YAP2. Levels of bound proteins and input are shown. C) Kinase assays of recombinant MBP-AM OT130 (preincubated with or without purified nonmuscle F-actin) and LATS2 kinase immunoprecipitated from HEK293 cells. Phosphorylated AM OT130 was detected using a phospho-S175-specific antibody. The levels of bound proteins and input are shown.
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-ABD 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 U20S cells blocked localization of coexpressed AMOT130 to F-actin, and both proteins localized to vesicles (Supplemental Figure S3D). We next tested 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 amounts 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 a 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濮Pontupel et al., 2011; Ferrandez et al., 2011; Sansores-Garcia et al., 2011; Wada et al., 2011; Zhao et al., 2012). Exam ple includes 1) F-actin depolymerization by latrunculin B or cytochalasin D; 2) am Samplamid of G protein-coupled receptors to affect the actin cytoskeleton in mouse cells (Micchetti et al., 2012; Mo et al., 2012; Yu et al., 2012); 3) type 2myosin inhibition, which affects F-actin stress filaments (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)/short hairpin RNA (shRNA) to knockdown AMOT, AMOTL1, and AMOTL2 in HEK293A and MCF10A cells (Supplemental Figure S4B). Although knockdown of individual angiomotin on target knockdowns, treatment of cell lines caused increased nuclear retention of YAP and maintenance of YAP activity after F-actin depolymerization, type 2 myosin inhibition, am Samplawid, and increased cell density in HEK293A and MCF10A cells (Figures 5, A–D, and Supplemental Figure 4C, C–F). Note that the effect of triple knockdown in HEK293A cells after latrunculin B treatment or serum starvation could be rescued by overexpression of AMOT130 or AMOTL2 (Figures 5, A and B) in HEK293A cells, triple angiomotin knockdown blocked cytoplasmic accumulation of YAP to a similar degree as IATSL2/2 knockdown after latrunculin B treatment but had a significantly stronger effect than IATSL2 knockdown after starvation (Figures 5, A and B). Combined knockdown of both IATSL2/2 and all three angiomotins caused an additive effect after latrunculin B treatment compared with knockdown of IATSL2/2 or the three angiomotins alone (Figure 5A). However, after serum starvation, combined IATSL2/2 and triple F-actin knockdown did not significantly enhance YAP nuclear retention compared with triple angiomotin knockdown alone (Figure 5B). The different relative effects of IATSL2 and angiomotin knockdown after latrunculin or serum starvation treatment could be explained if IATSLs and angiomotins respond somewhat differently to each stimulus. Collectively these results show that IATSL and angiomotins are a pair of effectors of various inputs that act through the F-actin cytoskeleton to affect YAP localization.

DISCUSSION

The F-actin cytoskeleton is a major regulator of the Hippo pathway target YAP, mediating signals triggered by substrate stiffness, cell density, and cell detachment, as well as signaling from G protein-coupled receptors (Dupont et al., 2011; Sansores-Garcia et al., 2011; Wada et al., 2011; Micchetti et al., 2012; Mo et al., 2012; Yu et al., 2012; Zhao et al., 2012). We show here that angiomotin proteins connect F-actin organization to YAP regulation. The AMOT130 protein binds purified F-actin in vitro, and we observe it on stress fibers in cells. 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 can bind F-actin in vitro, it will be important in future studies to determine whether AMOT130 can distinguish between different F-actin structures in vivo. A direct competition for binding to AMOT130 between F-actin and YAP appears to underlie the ability of F-actin to keep AMOT130 from binding and sequestering YAP in the cytoplasm. Angiomotins 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 is sequestered on F-actin structures and stabilizes a site that causes these structures, such as increased cell density, to release AMOT130, allowing it to bind and inhibit YAP.

This simple model may actually be more complex. For example, in overexpression studies, we observe that the phosphoamino acid form of AMOT130, which does not bind F-actin and has enhanced ability to keep YAP out of the nucleus, colocalizes with YAP in vesicular structures in the cytoplasm. This raises the possibility that membrane/vesicular localization could play an additional role in YAP regulation. It is worth noting that we only observe localization of endogenous phosphoAMOT130 and YAP to possible vesicular structures when F-actin is disrupted. In other situations, phosphoAMOT130 colocalizes with YAP at cell junctions. One explanation for these results is that overexpression of AMOT130-S175E may cause accumulation of vesicular intermediates that would normally be sent to the plasma membrane. Consistent with this notion, overexpression of AMOT80, a short form of AMOT lacking the

in single cells were quantified and correlated with endogenous YAP localization. The graph plots the average AMOT130 levels for individual cells (plotted based on AMOT levels) and are scored for those with one YAP in the nucleus than cytoplasm (N/C, solid symbols) or not (N/C + C/N, open symbol). B) Endogenous YAP and phosphoAMOT130 (p-AMOT130) staining in HEK193T cells with or without treatment with latrunculin B for 15 min. DNA is stained with DAPI. Bar, 20 mm. C) Single cells were transfected with the same AMOT130 plasmids as in A, as well as an 8XGFP-luciferase YAP-dependent promoter plasmid, and a plasmid with the SV40 promotor driving Renilla luciferase. The next day, cells were transfected with the indicated AMOT130 plasmids and renilla luciferase plasmids were norm ally normalized to the level of Renilla luciferase in each sample. Error bars indicate the SD between trilipid traps. Brackets on top of bars represent statistical significance (Student’s test, *p < 0.005, **p < 0.001). In all cases, the experiments were done in triplicate, and the error bars indicate the SD of the averages. D) IATSL2, YAP, and the indicated AMOT130 plasmids were transfected into HEK293 cells, and the levels of AMOT130, IATSL2, YAP, and phospho-YAP were analyzed by Western blotting. The experiments were done in triplicate, and error bars indicate the SD of the averages. E) 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-YAP2. 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, all 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). (C) Cells were grown in starvation media for 2 h, then fixed and stained for YAP localization (% of cells). (D) Cells were treated with cytochalasin D (CytoD) or starved (Starvation) and fixed and stained for YAP localization (% of cells). (E) Diagram illustrating the mechano-sensing pathway involving GPCR, AMOTs, Actin, and YAP.
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 in postnate of IATS for F-actin–dependent regulation of YAP. Dupont et al., 2011; Yu et al., 2012; Zhao et al., 2012; Amegna 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 sequesters 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; Amegna 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 vivo (Dailetal., 2013), and we show that the ability of IATS2 to phosphorylate AMOT130 in vivo 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; Dailetal., 2013; H Hate et al., 2013). We show that IATS phosphorylation blocks AMOT130 binding to F-actin, allowing it to bind to 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 W domain-containing E3 ubiquitin ligase AIP4, which can both stabilize AMOT130 and promote YAP degradation (Adler et al., 2013a,b). It seems 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 (H Hate et al., 2013) and have effects on the actin cytoskeleton (Dailetal., 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 LATS-independent mechanism for F-actin–dependent regulation of YAP. The LATS-dependent and LATS-independent mechanisms 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 LATS activity, such as cell–cell contacts (Kim et al., 2011), as was recently suggested (Amegna et al., 2013). Together, this work shows that F-actin, angiomotins, and IATS form a regulatory module that controls YAP in response to F-actin-dependent mechanisms or inputs that affect YAP localization or in conjunction with the actin cytoskeleton.

MATERIALS AND METHODS

Cell culture

Human HEK 293, HEK293A, Hela, and U2OS cells were grown in DM EMG (GIBCO, Grand Island, NY) supplemented with 10% (vol/vol) fetal bovine serum (GIBCO) and 1% (vol/vol) 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 IATS-mediated phosphorylation of angiomotins with P-32, HEK 293 cells were transfected in 12-well plates with IATS2, various angiomotin constructs, and IATS activators MST1, SAV, and MOB1, using Lipofectamine 2000 (Invitrogen). Forty hours after transfection, cells were lysed in im immunoprecipitation buffer 60 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, supplemented with 1 μg/ml protease inhibitor cocktail (Sigma-Aldrich), 100 mM sodium vanadate (Sigma-Aldrich), and 50 mM sodium fluoride (Sigma-Aldrich), and lysates were cleaned by centrifugation at 13,000 rpm for 10 min at 4°C. Protein lysate (200 μg) was processed for i mmunoprecipitation as described previously (Pannasistr et al., 2011). Both IATS2 and angiomotin proteins were immunoprecipitated together on the same bead. Kinase assays and Western blotting were carried out as previously described (Pannasistr et al., 2011).

For kinase assays in the presence of F-actin, IATS2–FLAG was transfected in HEK293 cells together with the β-actin construct, MST1 and MOB1. After 24 h, IATS2 was purified in phosphate buffer using anti-FLAG M2 antibody (Sigma-Aldrich) and magnetic protein G beads (Invitrogen) following the manufacturer’s directions. MAbase-binding protein MBP–AMOT130 was expressed and purified as described and eluted with 20 mM maltose in supplemented actin buffer 6 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 μM of F-actin (see Protein description, 5 μM final concentration) for 15 min at room temperature. Controls 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 nA. Exam ples in ages are shown. Brackets on top of bars represent statistical significance. Fisher exact, *p < 0.0005, **p < 0.005. C) Lentiviral infection was used to introduce either control siRNA (directed against luciferase) or siRNA against all three angiomotins AMOT130, AMOT1 and AMOT2; triple knockdown) into MCF10A cells. Sixty hours after infection, cells were left untreated, treated with tyrosine kinase D. Cytochalasin D (CytD), or treated with amn for an additional 12 h. Cells were then fixed and stained for endogenous YAP. YAP localization was scored as nA. Exam ples in ages are shown. (D) HEK293A cells were transfected to be with control or a combination of AMOT130, AMOT1, and AMOT2 siRNA (see Materials and Methods). Cells were fixed after 48 h and stained for endogenous YAP. YAP localization was scored as nA. (E) M Kd, knockdown; N, nucleus. E) M odellof F-actin–regulated angiomotin (AMOT) inhibition of YAP.
Luciferase assays were performed in U2OS and HeLa cells 24 h after transfection. All transfactions were performed in 12-well plates using Lipofectamine 2000 and a combination of 300 ng of T7-MT-Luc (Promega), Addgene, Cambridge, MA, 20 ng of pRL-SV40P (Promega) and the described AMOT130 plasmid (300 ng for U2OS and 25 ng for HeLa cells). Cells were harvested 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/PBS supplemented with 100 ng/ml cholera toxin (Sigma-Aldrich) and 1% fetal bovine serum. Latrunculin B and cytochalasin D were used at 1 mM for 1 h, except for the phospho-AMOT130/YAP staining (Figure 4D), for which cells were incubated for 0.15 mM for 30 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 added to the secondary antibody solution when appropriate. After fixation, cells were permeabilized with 0.1% Triton X-100 and 5% normal goat serum (Invitrogen). Latrunculin B and cytochalasin D were subsequently incubated with appropriate primary antibodies for 1-2 h at room temperature. They were then washed three times in PBS with 0.1% Triton X-100 and incubated with Alexa Fluor-conjugated secondary antibodies (Molecular Probes, Grand Island, NY) for 1 h at room temperature. 4,6-Diamidino-2-phenylindole (DAPI) staining and localization studies were performed in a 12-well format. The various antibodies used were rabbit-anti YAP (sc15407), mouse anti-YAP (c10199), rabbit anti-Myc (c879), mouse anti-Myc 9E10 (c56), mouse anti-GFP 9996, mouse anti-AMOT B-4 (c-166924), and goat anti-AMOTL2 (2501) from Santa Cruz Biotechnology (Dallas, TX). Myosin IIA was purchased from Cell Signaling Technology (9403; Beverly, MA). The rabbit anti-AM OT antibody was generated by the Fernandes lab (CHUQ-CHUL Research Center, Université Laval, Québec City, Canada). Rabbit anti AMOT L2 was provided by Anthony Schmitt (Pennsylvania State University, State College, PA). 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 for 1 h. Pellets were resuspended in the same volume as the supernatants and boiled in SDS-PAGE sample 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 (Paramasivam et al., 2011). A AMOT130, AMOT1L, and AMOT2 constructs were expressed from pCDNA4-MycHis. Large deletion mutants in AMOT130, AMOT1L, and AMOT2 were constructed using PCR followed by subcloning. Point and small deletion mutations in AMOT130 and AMOT2 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. The various antibodies used were rabbit-anti YAP (sc15407), mouse anti-YAP (c10199), rabbit anti-Myc (c879), mouse anti-Myc 9E10 (c56), mouse anti-GFP 9996, mouse anti-AMOT B-4 (c-166924), and goat anti-AMOTL2 (2501) from Santa Cruz Biotechnology (Dallas, TX). Myosin IIA was purchased from Cell Signaling Technology (9403; Beverly, MA). The rabbit anti-AM OT antibody was generated by the Fernandes lab (CHUQ-CHUL Research Center, Université Laval, Québec City, Canada). Rabbit anti AMOT L2 was provided by Anthony Schmitt (Pennsylvania State University, State College, PA). 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 for 1 h. Pellets were resuspended in the same volume as the supernatants and boiled in SDS-PAGE sample buffer. Protein samples were subjected to SDS-PAGE and western blotting with the specified antibodies.

Antibodies

Mouse anti-tubulin and mouse anti-FLAG (M2) were purchased from Sigma-Aldrich. The rabbit-anti YAP (sc15407), mouse anti-YAP (c10199), rabbit anti-Myc (c879), mouse anti-Myc 9E10 (c56), mouse anti-GFP 9996, mouse anti-AMOT B-4 (c-166924), and goat anti-AMOTL2 (2501) were from Santa Cruz Biotechnology (Dallas, TX). Myosin IIA was purchased from Cell Signaling Technology (9403; Beverly, MA). The rabbit anti-AM OT antibody was generated by the Fernandes lab (CHUQ-CHUL Research Center, Université Laval, Québec City, Canada). Rabbit anti AMOT L2 was provided by Anthony Schmitt (Pennsylvania State University, State College, PA). 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 for 1 h. Pellets were resuspended in the same volume as the supernatants and boiled in SDS-PAGE sample buffer. Protein samples were subjected to SDS-PAGE and western blotting with the specified antibodies.

In vitro protein-binding assays

AMOT130 and AMOT130-S175E were cloned in pDEST-MBP (provided by Maarten Wiaux at the Gateway [Invitrogen] standard procedure. 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 maliose beads (NEB, Ipswich, MA) in phosphate buffer 50 mM NaH2PO4, 150 mM NaCl, 10 mM isopropyl-β-D-thiogalactoside, 0.1% Triton, and 1 mM phenylmethylsulfonyl fluoride) following the manufacturer's directions. Expression of glutathione S-transferase (GST)-YAP2 (pGEX-5X-2 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 cells were purchased as part of the Actin Binding Protein Kit (Cytoskeleton) and were polymerized for 1 h at 25°C following the manufacturer's directions. For the in vitro pulldown experiments, beads-bound AMOT130 and AMOT130-S175E were incubated for 30 min at room temperature with either GST-AMOT130 and/or 50 nM F-actin in phosphate buffer containing 2 mM ATP and 2 mM MgCl2 to keep F-actin stable. Actin binding protein kit manual. Competition assay was 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-AMOT130 or F-actin was 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 coimmunoprecipitation experiment, 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 for 1 h. Pellets were resuspended in the same volume as the supernatants and boiled in SDS-PAGE sample buffer. Protein samples were subjected to SDS-PAGE and western blotting with the specified antibodies.

sRNA/shRNA transfection

Knockdowns in HEK293A cells were performed using 30 nM control sRNA or SMARTpool sRNA (Dharmacon, Lafayette, CO) and 3 µl of RNA MAX Lipofectamine (Invitrogen). Cells were cultured for 48 h after transfection. The only exceptions were experiments with cells at high densities, for which sRNAs were transfected twice at 40 nM (second transfection after 24 h), and cells were transfected for 2 h of the first transfection. For assessing experiments, plasmids expressing expression vectors were transfected 24 h after of knockdown with Lipofectamine 2000. Slncing reagents were as follows. Controls sRNA (fully licensed, 5'-CUAGCGCGGAAUCUGA3', labeled as to G12L), AMOT SMARTpool sRNA targeting both AMOT80 and AMOT130; M-015417), AMOT SMARTpool sRNA LATS2 SMARTpool sRNA (M-0157959), AMOT SMARTpool sRNA LATS2 SMARTpool sRNA (M-013322), LATS2 SMARTpool sRNA (M-006632), and LATS2 SMARTpool sRNA (M-003865). MCF10A cells were transfected using lentiviral infection of shRNAs, and cells were collected after 3 d. For the studies with AMOT1L knockdown alone, MCF10A cells were transfected with constructs for stably knocking down AMOT1L and control luciferase were used (Paramasivam et al., 2011). To generate a triple knockdown, stable AMOT1L
knockdown cells were infected with a combination of AMOT130 and AMOTL2 lentiviral supernatants. At the same time, stable control cells were infected with control lentiviral supernatants. A control V5a lentiviral supernatant was generated by the shRNA Com Facility, University of Massachusetts Medical School (Worcester, MA), to target GCCAGGAAGAACTGTTG AMOTL1 or TGTTGAG-TATCTCCATT AMOT130.

Realtime q 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 primer using SuperScript II Reverse Transcriptase (Invitrogen). Realtime q quantitative PCR was performed using KAPA SYBR FastMaster in Unisensmart 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, CTCCTGCACCACCACTCTTG; GAPDH-R, GGGCCATCCACAGTCTTCTG; AMOT-F2, ACTACCACCACCTCCAGTCA; AMOT-R2, ACAAGTGACCACTTGGCTC; AMOTL2-F1, GCTACTGGGGTAGCAACTGA; and AMOTL2-R1, GGAAGCAGTGAGGAACTGAA. AMOT, AMOTL2, and AMOTL2 primers were ordered from RealTime Primers (Elkins Park, PA).

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