Angiomotins link F-actin architecture to Hippo pathway signaling

Sebastian Mana-Capelli, Mungan Paramasivam, Shubham Dutta, and Dannel McCollum

Department of Biochemistry and Molecular Pharmacology and Program in Cell Dynamics, University of Massachusetts Medical School, Worcester, MA 01605

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 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 (Miller et al., 2012; Mo 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). In flies, angiomotin acts to mediate the effects of F-actin on YAP localization, allowing cells to make developmental and proliferative decisions based on diverse inputs that regulate actin architecture.
results

The N-terminal Hippo pathway regulatory domain of angiomotins contains an actin-binding motif. Overexpression of the long isoform of AMOT (AMOT130) causes formation of large F-actin bundles that also contain AMOT130 (Ernkvist et al., 2008; Dai et al., 2013; Figure 1A). When expressed at lower levels, AMOT130 localizes as puncta on stress fibers but does not cause obvious actin bundling (Figure 1B). To determine the significance of AMOT130 localization to the actin cytoskeleton, we sought to identify mutants defective in actin localization and bundling. Deletion analysis revealed that the actin localization domain was contained within an 100-aa region of all three angiomotin proteins (Figure 1, A, C, and D, and Supplemental Figure S1A). By deleting individual blocks of conserved sequence within this region, we found that actin localization required a short motif (e.g., AMOT130 residues 169-178; Figure 1, C and D). Deletion of this region in full-length AMOT130 (AMOT130-AB) disrupts actin localization and bundling activity. Note that the AMOT130-AB mutant and other forms of AMOT130 that cannot bind F-actin localize to vesicular structures (see Discussion), as observed for AMOT80 (Heber 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 phospho-tyrosine 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). Therefore, LATS2 and its phosphorylation site play critical roles in regulating actin binding and localization of angiomotins.
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 < 0.0001). 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 colocalize with actin filaments (Figure 2D). In contrast, phospho-AMOT130 (analyzed with phospho-specific 175–specific antibodies; Hikata et al., 2013) did not colocalize with F-actin fibers and instead was 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-mimetic AMOT130–AB with deletions in either the YAP-binding motifs or the LAD demonstrated that the enhanced ability of AMOT130–AB to promote YAP localization to the cytoplasm depends mostly on the L/PPXY motifs, with the LAD making only a minor contribution (Figure 4B). This suggests that F-actin binding primarily interferes with AMOT130 binding to YAP. To address this question, we made AMOT130 mutants that were specifically defective at activating LATS2 or binding to 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 WW domains of YAP (Chan et al., 2011; Wang et al., 2011; Zhao et al., 2011; Dupont et al., 2008). Because mutation of residues 13–27 abolished the ability of AMOT130 to activate LATS2 (Supplemental Figure S3A), we made AMOT130 mutants that were specifically defective at activating LATS2 or binding to YAP. To disrupt interaction between AMOT130 and YAP, we mutated two L/PPXY motifs in AMOT130 that are known to mediate interaction between AMOT130 and YAP. When we assayed transcription from a synthetic YAP-dependent promoter in 293T cells, both AMOT130 and YAP were coexpressed in the nucleus. Wt-type AMOT130 and AMOT130-S175A were able to cause limited translocation of YAP to the cytoplasm in addition to high AMOT130 expression levels (Figure 4C). Of interest, the AMOT130-S175A mutant was less effective than wt-type AMOT130 at bringing YAP to the cytoplasm. In contrast, the mutants that could not bind F-actin (AMOT130–S175E or AMOT130–S175A) 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 (Figure 4A). These experiments show that F-actin binding antagonizes the ability of AMOT130 to inhibit YAP nuclear localization and function.

When we assayed transcription from a synthetic YAP-dependent promoter in 293T cells, both AMOT130 and YAP were coexpressed in the nucleus. Wt-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 wt-type AMOT130 at bringing YAP to the cytoplasm. In contrast, the mutants that could not bind F-actin (AMOT130–S175E or AMOT130–S175A) were much more effective at shifting YAP to the cytoplasm (Figure 4A). These experiments show that F-actin binding antagonizes the ability of AMOT130 to inhibit YAP nuclear localization and function.
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 are shown. D) Untreated and Latrunculin B treated samples. E) Normalized YAP activity is shown for each condition. F) Western blot analysis of phosphorylated YAP, YAP, LATS2, and AMOT130. G) Schematic representation of the PD (AMOT130) and YAP2 pathways.
could allow F-actin levels to modulate the ability of AMOT130 to bind to YAP. Consistent with this idea, overexpression of YAP in U20 S 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 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 treatment that perturb F-actin (Supplemental Figure S4A) cause YAP to exit the nucleus Dupont et al., 2011; Pernandez et al., 2011; Sansores-Garcia et al., 2011; Wada et al., 2011; Zhao et al., 2012). Examples 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 M Berretta, 2012; Mio et al., 2012; Yu et al., 2012); 3) type II myosin inhibition, which affects F-actin stress fibers Dupont et al., 2011; and 4) increased cell density Dupont et al., 2011). We found that angiomotins and LATS are required for upregulation 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 orthologs had mixed effects, knockdown of all three caused decreased retention of YAP and maintenance of YAP activity after F-actin depolymerization, type II myosin inhibition, serum withdrawal, and increased cell density in HEK293A and MCF10A cells (Figures 5, A-D, and Supplemental Figure S4, C-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 AMOTL2; Figures 5, A and B). In HEK293A cells, 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 after serum starvation. Figure 5, A and B). Combined knockdown of both LATS1/2 and all three angiomotins caused an additive effect after latrunculin B treatment compared with knockdown of LATS1/2 or any of the three angiomotins alone (Figure 5A). However, after serum starvation, combined LATS1/2 and triple angiomotin knockdown did not significantly enhance YAP nuclear retention compared with triple angiomotin knockdown alone (Figure 5B). The different relative effects of LATS and angiomotin knockdown after latrunculin or serum starvation treatment could be explained if LATS and angiomotin orthologs respond somewhat differently to each stimulus. Collectively these results show that LATS and angiomotins are in parallel pathways 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; M Berretta, 2012; Mio 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 types of F-actin structures in vivo. A direct competition between AMOT130 and F-actin appears to underlie the ability of F-actin to keep AMOT130 from binding and sequestering YAP in the cytoplasm. Angiomotins are major regulators 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 stimuli that cause loss of these structures, such as increased cell density, result in release of 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 phosphomimetic 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 vesicle localization could play an additional role in YAP regulation. It is worth noting that we only observe localization of endogenous phospho-AMOT130 and YAP to possible vesicular structures upon F-actin disruption. In other situations phospho-AMOT130 co-localizes 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 on to the plasma membran. Consistent with this notion, overexpression of AMOT80, a short form of AMOT lacking the...
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) YAP localization (% of cells) was also analyzed under conditions of starvation and cytochalasin D treatment. D) YAP localization was assessed under conditions of untreated, cytochalasin D, and starvation treatments. E) A schematic diagram illustrating the proposed mechanisms for the regulation of YAP by angiomotins and LATS in response to F-actin disturbance.
F-actin-binding domain, causes accumulation of large endosomal-like compartments (Heber et al., 2010). In situ studies will be important to determine the mechanism of localization of AM OT130-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), Amezquita 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 synaptic 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; Amezquita et al., 2013). Our work indicates that activated IATS can also act through angiomotins to inhibit YAP. IATS phosphorylation of AM OT130 is enhanced by F-actin disruption in vitro (Dai et al., 2013), and we show that the ability of IATS to phosphorylate AM OT130 in vivo is increased in the absence of F-actin. From this study, as well as from several recent reports, it is clear that LATS phosphorylation of AM OT130 inhibits its ability to bind F-actin (Adler et al., 2013b; Chan et al., 2013; Dai et al., 2013; He et al., 2013). We show that IATS phosphorylation blocks AM OT130 binding to F-actin, allowing it to bind to YAP and sequester it in the cytoplasm. LATS phosphorylation of AM OT130 appears to have additional functions. A recent study indicates that AM OT130 phosphorylation could also enhance AM OT130 binding to the W domain-containing FAM ubiquitin ligase AP4, which can both stabilize AM OT130 and promote YAP degradation (Adler et al., 2013a,b). It remains to be determined whether AP4, like YAP, directly competes with F-actin for binding to AM OT130. Recent studies also suggest that AM OT130 phosphorylation by LATS could enhance the AM OT130-LATS interaction (He 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 AM OT130 in addition to its well-characterized function in phosphorylating YAP (Figure 5B).

The competition between F-actin and YAP for binding to AM OT130 could also provide a LATS-independent mechanism for F-actin-dependent regulation of YAP. The LATS-dependent and -independent 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 LATS activity, such as cell-cell contacts (He et al., 2013), as was recently suggested (Amezquita et al., 2013). Together, this work shows that F-actin, angiomotins, and IATS form a regulatory module that controls YAP in response to different inputs such as changes in cell density, substrate stiffness, and G protein-coupled receptor signaling (Adler et al., 2012).

**Materials and Methods**

**Cell culture**

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 and 1% (vol/vol) penicillin/streptomycin (Invitrogen). Human breast epithelial MCF10A cells were cultured in MEBM BulletKit Low Attachment (Lonza, Hopkinton, MA) with all additives except for the gentamicin/sodium polysulfate (Invitrogen). Hum an mammary epithelial MCF10A cells were cultured in MEBM BulletKit Low Attachment (Lonza, Hopkinton, MA) with all additives except for the gentamicin/sodium polysulfate (Invitrogen). Actin cells 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 F-32, HEK 293 cells were transfected in 12-well plates with IATS2, various angiomotin constructs, and IATS activators MST1, ST1, and MOI. After 24 h, cells were lysed in RIPA buffer supplemented with 100 mM sodium vanadate (Sigma-Aldrich), and 50 mM sodium fluoride (Sigma-Aldrich), and lysates were cleaved by centrifugation at 13,000 rpm for 10 min at 4°C. Protein lysates (100 µg) were processed for RIPA buffer as described previously (Paramasivam 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 (Paramasivam et al., 2011).

**For kinase assays in the presence of F-actin, IATS2-FLAG was transfected in HEK293 cells together with its activators, MST1 and MOI. 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-AM OT130 was expressed and purified as described and eluted with 20 mM maltose in supplemented actin buffer (30 M 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 AM OT130 (10 µl, 0.5 µg) was then incubated with or without 10 µl of F-actin (see Materials and Methods, 5 µM final concentration) for 15 min at room temperature. Control reactions were taken to 20 µl with supplemented actin buffer. For kinase reactions, the AM OT130/F-actin mix was added to IATS2-bound beads premixed 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-AM OT130 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 combination of 300 ng of T7E1-Luc (4615; Addgene, Cambridge, MA), 20 ng of pRL-SV40 (described as SV40, 27163; Addgene), and the described AM OT130 plasmid 900 ng for U2OS and 25 ng for HeLa cells. Cells lysates were generated and reactions performed following directions described in the Dual-Luciferase Reporter Assay System from 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 with 10% fetal bovine serum and then incubated for 1 h with 1 µM AM OT130 and 1% penicillin and streptomycin (Invitrogen). Latrunculin B and cytochalasin D were used at 1 µM each, except for the phospho-AMOT130/YAP stainings (Figure 4D), for which cells were incubated 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 used at 25 µM for 1 h.

In vivo two-color assay

U2OS, HeLa, and MCF10A cells cultured on coverslips were fixed in phosphate-buffered saline PBS/0.1% formaldehyde for 10 min and permeabilized in 0.1% Triton X-100 and 5% nonfat milk (blocking buffer) 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 for 1 h at room temperature. The specimens were mounted on slides using Vectashield (Vector Laboratories, Burlingame, CA) and viewed using fluorescent microscopy (Olympus IX71; Olympus, Tokyo, Japan) or a cooled charged-coupled device camera (ORCA-ER; Hamamatsu, Japan). Myosin IIa was purchased from Invitrogen.

Sources for plasmids used in this study were described previously (Paum asakam et al., 2011). A LATS2 construct was expressed from pcDNA4-Myc-His. Large deletion mutants in AMOT130, AMOTL1, 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. The various anti–GFP constructs used in this study were described previously (Schneider et al., 2012).

Knockdowns of AMOTL2

For rescuing experiments, plasmids for protein expression were transfected after 24 h of knockdown with Lipofectamine 2000 and a combination of 300 ng of GTIIC-AMOTL2 plasmid (300 ng for U2OS and 25 ng for HeLa cells). 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 72 h after the first transfection. For rescuing experiments, plasmids for protein expression were transfected after 24 h of knockdown with Lenti-X expressing constructs. Knockdowns and siRNA/shRNA transfection

Knockdowns in HEK293A cells were performed using 30 nM control siRNA or SMARTpools (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 siRNAs were transfected twice at 40 nM (second transfection after 24 h), and cells were fixed after 72 h of the first transfection. For rescuing experiments, plasmids for protein expression were transfected after 24 h of knockdown with Lipofectamine 2000. Silencing agents were as follows: ControlsiRNA (sc789), AMOT130 siRNA 5′-CGUACGCGGAAUACUUCGA-3′ (referred to as G12), AMOT130 SMARTpool siRNA targeting both AMOT130 (M-015417), AMOT130 SMARTpool siRNA (M-015417), AMOT130 SMARTpool siRNA (M-013232), LATS1 SMARTpool siRNA (M-006362), and LATS2 SMART pool siRNA (M-003865). MCF10A cell knockdowns were done using lentiviral infection of siRNA, and cells were collected after 3 d. For the studies with AMOTL2 knockdown alone, MCF10A cells treated with stable constructs for stably knocking down AMOTL2 and control (luciferase) were used (Paum asakam et al., 2011). To generate a triple knockdown, stable AMOTL2

sRNA/shRNA transfection

Knockdowns in HEK293A cells were performed using 30 nM control siRNA or SMARTpools (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 siRNAs were transfected twice at 40 nM (second transfection after 24 h), and cells were fixed after 72 h of the first transfection. For rescuing experiments, plasmids for protein expression were transfected after 24 h of knockdown with Lipofectamine 2000. Silencing agents were as follows: ControlsiRNA (sc789), AMOT130 siRNA 5′-CGUACGCGGAAUACUUCGA-3′ (referred to as G12), AMOT130 SMARTpool siRNA targeting both AMOT130 (M-015417), AMOT130 SMARTpool siRNA (M-013232), LATS1 SMARTpool siRNA (M-006362), and LATS2 SMART pool siRNA (M-003865). MCF10A cell knockdowns were done using lentiviral infection of siRNA, and cells were collected after 3 d. For the studies with AMOTL2 knockdown alone, MCF10A cells treated with stable constructs for stably knocking down AMOTL2 and control (luciferase) were used (Paum asakam et al., 2011). To generate a triple knockdown, stable AMOTL2
knockdown cells were infected with a combination of AMOT130 and AMOTL1 lentiviral supernatants. At the same time, stable control cells were infected with control lentiviral supernatants. A control V64 cells were generated by the shRNA Core Facility, University of Massachusetts Medical School (Worcester, MA), to infect target mRNA levels were measured relative to control cells were infected with control viral supernatant as a control.

Viral supernatants were generated by the shRNA Core Facility, University of Massachusetts Medical School (Worcester, MA), to infect target mRNA levels were measured relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels.

The following primers were used:
- **GAPDH-F**, CTCCTGCACCCCACTCCTG, and **GAPDH-R**, GGGGTCACTCACTCTTGGTCTF; **CTGF-F**, AGGAGTTGGGTTGCGGCA, and **CTGF-R**, CCAGGCCCCAGTTGCTCTA; **AMOT-F1**, GCAGAGAACTGAGGA, and **AMOT-R2**, ACAAGGTGACGACTCTCTGC; **AMOTL1-F1**, AAATGTGGTGGGAATTCTGCAGAAGCATGAG; and **AMOTL1-R1**, AAATGTGGTGGGAATTCTGCAGAAGCATGAG. AMOT, AMOTL1, and AMOTL2 primers were ordered from Real Time Prime rs (Elkins Park, PA).

**ACKNOWLEDGMENTS**

We thank Clark Wells and Bin Zhao for communication of unpublished results; Anthony Schmidt, Maria Fernandes, and Hiroshi Sasaki for their bodies; Elizabeth Luna for technical advice; and Peter Pyckich for comments on the manuscript. This work was supported by National Institutes of Health Grant GM058406-14 to D.M.

**REFERENCES**


**ACKNOWLEDGMENTS**

We thank Clark Wells and Bin Zhao for communication of unpublished results; Anthony Schmidt, Maria Fernandes, and Hiroshi Sasaki for their bodies; Elizabeth Luna for technical advice; and Peter Pyckich for comments on the manuscript. This work was supported by National Institutes of Health Grant GM058406-14 to D.M.

**REFERENCES**


