Actin-binding and Cell Proliferation Activities of Angiomotin Family Members Are Regulated by Hippo Pathway-mediated Phosphorylation*

Siew Wee Chan†1, Chun Jye Lim†, Fusheng Guo†, Ivan Tan†, Thomas Leung§, and Wanjin Hong†§2

Background: LATS kinase, one of the core kinases of Hippo pathway, phosphorylates and inactivates the downstream coactivator YAP/TAZ.
Results: The angiomotin (Amot) family members are phosphorylated by LATS kinase.
Conclusion: Phosphorylation of Amots by LATS kinase inhibits actin-binding, stabilizes Amot, and inhibits cell proliferation.
Significance: Besides phosphorylating YAP/TAZ, LATS kinase may phosphorylate other components of the Hippo pathway.

Whether the Hippo pathway has downstream targets other than YAP and TAZ is unknown. In this report, we have identified angiomotin (Amot) family members as novel substrates of Hippo core kinases. The N-terminal regions of Amot proteins contain a conserved HRXXS consensus site for LATS1/2-mediated phosphorylation. Phospho-specific antibodies showed that Hippo core kinases could mediate phosphorylation of endogenous as well as exogenous Amot family members. Knockdown of LATS1 and LATS2 endogenously reduced the phosphorylation of Amots detected by the phospho-specific antibodies. Mutation of the serine to alanine within this HRXXS site in Amot and AmotL2 established that this site was essential for Hippo core kinase-mediated phosphorylation. Wild-type and non-phosphorylated Amot (Amot-S175A) were targeted to actin filaments, whereas phospho-mimic Amot (Amot-S175D) failed to be localized with actin. Overexpression of LATS2 caused dissociation of Amot from actin but not Amot-S175A. Mapping of the actin-binding site of Amot showed that serine 175 of Amot was important for the actin-binding activity. Amot-S175A promoted, whereas Amot and Amot-S175D inhibited, cell proliferation. These results collectively suggest that the Hippo pathway negatively regulates the actin-binding activity of Amot family members through direct phosphorylation.

The Hippo pathway plays an important role in organ size control via regulating cell proliferation, differentiation, and apoptosis (for reviews, refer to Refs. 1, 2, and 7). There is growing evidence that deregulation of the Hippo pathway often leads to different types of cancers (3, 4). The pathway comprises of three major parts: the upstream regulators, the Hippo core kinase complex, and the downstream effectors. The major components of the Hippo core kinase complex as well as the downstream targets are conserved between flies and mammals, whereas the upstream regulators of the pathway are not well conserved throughout evolution (5).

The core kinase complex of mammals consists of four components, two kinases (MST1/2 and LATS1/2)3 and two adaptors (WW45 for MST1/2 and MOB1 for LATS1/2). When activated by upstream signals, LATS1/2-MOB1 phosphorylates the downstream effectors YAP and TAZ. Whether the Hippo pathway executes its regulation via other possible downstream substrates is an open question.

Angiomotin (Amot) was originally identified as a protein interacting with the angiogenesis inhibitor angiostatin (6). There are three members in the Amot family: Amot (which exists as Amot-p130 or Amot-p80), angiomotin-like 1 (AmotL1), and angiomotin-like 2 (AmotL2) (7). Amots are characterized by central coiled coil domains and C-terminal PDZ-binding regions (7). Amot-p130 and Amot-p80 are generated by alternative splicing, with the latter lacking the N-terminal 409 amino acids of the former (8).

Amot family members are implicated as both negative and positive growth regulators, depending on cellular context, but the molecular basis for the differential role of Amots is unknown. Amot-p80 has been shown to have the ability to promote endothelial cell migration and tube formation and has a role in angiogenesis (6, 9). It is also expressed at higher level in breast cancer tissues (10). Although Amot-p80 promotes the migration of endothelial cells, Amot-p130 does not. The N-terminal extension of Amot-p130 is important for interacting with actin filaments and maintaining cell shape and cell-cell junctions (8, 11, 12). Recently, Amot family proteins have been identified as interacting proteins of YAP and TAZ and act to antagonize the function of YAP and TAZ (13–15). However, there is also evidence showing that Amots are positively regulating cell growth and proliferation. Depletion of Amot in neurofibroma-

*This work was supported by the Agency for Science, Technology, and Research (A*STAR), Singapore.
†To whom correspondence may be addressed: Institute of Molecular and Cell Biology, 61 Biopolis Drive, Singapore 138673, Republic of Singapore. Tel.: 658-69602; Fax: 677-91117; E-mail: mcbhvj@imcb.a-star.edu.sg.
§1To whom correspondence may be addressed: Institute of Molecular and Cell Biology, 61 Biopolis Drive, Singapore 138673, Republic of Singapore. Tel.: 658-69606; Fax: 677-91115; E-mail: mcbhajv@imcb.a-star.edu.sg.

3 The abbreviations used are: LATS, large tumor suppressor kinase; YAP, Yes-associated protein; TAZ, transcriptional co-activator with PDZ-binding motif; MOB, Mps one binder kinase activator-like; Amot, angiomotin; MDCK, Madin-Darby canine kidney.
Amots as Hippo Pathway Substrates

In this study, we show that Amot proteins are substrates of the Hippo pathway. The LATS1/2 kinase of the Hippo core complex was able to phosphorylate Amots, and the phosphorylation of Amots by LATS1/2 leads to their dislocation from actin at the junction of epithelial cells and relocation to the cytoplasm. Phospho-mimic mutants of Amots negatively regulate cell proliferation. Our results demonstrate that the Hippo core kinase complex, besides phosphorylating the downstream YAP and TAZ, is able to phosphorylate other important Hippo pathway components and adds another layer of regulation of growth and proliferation.

EXPERIMENTAL PROCEDURES

Cell Culture—MCF10A and HEK293 cells have been described previously (13, 17–19). MDCK cells were from the laboratory of Dr. Hunziker (IMCB, Singapore) and maintained in DMEM supplemented with 10% fetal bovine serum. 293FT cells were purchased from Invitrogen.

Plasmids—HA-Amot and HA-AmotL1 were described previously (13). Full-length human cDNA clones of AmotL2, MST2, WW45, LATS1, LATS2, and MOB1 were purchased from OriGene. The HA epitope was tagged at the N-terminal of AmotL2 and cloned into pCIneo (Promega). MST2 is either Myc- or FLAG-tagged at the N terminus. WW45, STK38, and STK38L were Myc-tagged at the N terminus. LATS1 and LATS2 were HA epitope-tagged at the N terminus. All Hippo core kinase components were cloned into pCIneo. The lentiviral vector pLVX-Puro from Clontech was used to express HA-AmotL2 and FLAG-LATS2. The pBabe-puro retroviral vector was used to clone HA-Amot and mutants. The N-terminal regions of Amot and the mutant (S175A) encompassing amino acids 1–409 or the N-terminal fragments of Amot were cloned into pGEX-4T to make GST fusion proteins. The LATS2 core kinase-dead mutants HA-LATS2-KD-S872A and HA-LATS2-KD-T1041A were made by altering the serine residue at 872 into a glycine residue. The other mutants were made by altering specific amino acid residues 1–409 or the N-terminal fragments of Amot and the mutant (S175A) encompassing amino acids 1–409 or the N-terminal fragments of Amot into pGEX-4T to make GST fusion proteins. The LATS2 kinase-dead mutants HA-LATS2-KD-S872A and HA-LATS2-KD-T1041A were made by altering the serine residue at 872 and the threonine at 1041 to alanine, respectively.

Antibodies—Anti-FLAG and anti-actin antibodies were purchased from Sigma. Anti-HA was from Bethyl, and anti-Myc was from Cell Signaling Technology and Santa Cruz Biotechnology. Phospho-YAP antibody was from Cell Signaling Technology. Rabbit anti-phospho-Amot and rabbit anti-phospho-AmotL2 antibodies were custom-made by Genemed Synthesis, Inc. The peptide sequence of Amot used for raising phospho-Amot antibody was HCGLRLDKQGHVRSLS-(PO3H2)ERLQMQLSATL-OH, and the peptide sequence used for raising phospho-AmotL2 antibody was H-CALRELHGHRVSLSP(O3H2)ERLLQLSLER-OH. ZO-1 antibody was from Zymed Laboratories Inc..GST antibody was from Santa Cruz Biotechnology.

Reagents—Phos-tag was purchased from Wako Pure Chemical Ind., Ltd., and phos-tag SDS-PAGE was made according to the instructions of the company. Approximately 50 μg of total cell lysate was loaded onto 6.5% SDS-PAGE with 25 μM of phos-tag gel, and the proteins were transferred to a blotting PVDF membrane according to the recommendation of the company. Rhodamine phalloidin for F-actin staining in cells, Alexa Fluor 488, and Alexa Fluor 594 anti-rabbit secondary antibody for immunofluorescence were purchased from Molecular Probes. SMARTpool siRNAs of human LATS1 and LATS2 were from Dharmacon. λ phosphatase was from New England Biolabs. Transwell permeable support 0.4-μm polycarbonate membranes, 12-mm inserts, and 12-well plates were from Costar. Transfection, Western blotting, and immunoprecipitation have been described previously (13, 17–19).

λ-Phosphatase Treatment of Cell Lysates—Cells were lysed with lysis buffer (150 mM NaCl, 50 mM Tris (pH 7.4), 0.05% TX-100, and protease inhibitor mixture (Roche)). 50 μg of lysate supplemented with 2 mM MnCl2 was incubated with 200 units of λ-phosphatase from New England Biolabs for 30 min before immunoblotting.

Colony Formation Assay—10,000 MCF10A cells stably expressing a vector control, Amot, or mutants were cultured on 6-well plates. After 5 days, cells were washed with PBS and fixed with 10% methanol and 0.1% acetic acid for 30 min before staining with crystal violet (0.05% in 20% ethanol) for 3 h. Excess dye was washed away with water, and the stained cells were air-dried. Experiments were done in triplicates.

Virus Generation and Infection—MCF10A cells stably expressing Amot and mutants were obtained through retroviral infection. HA-Amot and HA-Amot mutants were cloned into the retroviral vector pBABE-puro, and the infection method has been described previously (13, 17–19). MDCK cells stably expressing LATS2 either with HA-AmotL2 or mutants were obtained through lentiviral-mediated infection. Lentiviruses were produced in 293FT cells using ViraPower lentiviral expression systems from Invitrogen. 1 ml of lentivirus supernatant (supplemented with 6 μg/ml Polybrene) produced from 293FT cells, cultured on a 10-cm plate, was used to infect MDCK cells seeded on 35-mm dishes overnight. Cells were recovered in fresh complete medium for 24 h before being selected with 1 μg/ml of puromycin for 5 days.

Immunofluorescence—Immunofluorescence has been described previously (18). 300,000 MDCK cells expressing HA-Amot, HA-Amot-S175A, and HA-Amot-S175D were seeded on a 12-well plate with 12-mm inserts of 0.4-μm Transwell filters (Costar) on day 1. On day 3, the cells were fixed with cold methanol (2.5 min, −20 °C) and permeabilized with Triton X-100 (0.1% in PBS). The permeabilized cells were incubated with the indicated antibodies, and immunofluorescence was performed as described previously. The filter was cut out, mounted with Clear-mount (with Tris buffer) from Electron Microscopy Sciences, and dried overnight before the images were acquired by confocal laser scanning microscope (Zeiss).

In Vitro Kinase Assay—HEK293 cells were transfected with HA-LATS2 and HA-LATS2-KD-S872A. 24 h after transfection, the cells were lysed with lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Nonidet P-40, Halt phosphatase (Pierce), and protease inhibitor mixture (Roche)) and immunoprecipitated with anti-HA antibody. The immunoprecipitates were washed four times with lysis buffer and twice with kinase buffer (20 mM Hepes (pH 7.6), 20 mM MgCl2, 5 mM NaF, 1 mM
sodium orthovanadate, 20 mM DTT, 20 mM β-glycerophosphate, and 20 μM ATP) before being used for the kinase assay.

In each kinase reaction, recombinant GST or GST fusion proteins of the N-terminal region of Amot and Amot-S175A were added to the reaction with immunoprecipitated LATS2 and LATS2-KD in kinase buffer supplemented with 10 μCi of γ-[32P]ATP. The reactions were done at 30 °C for 30 min, terminated with SDS sample buffer, and finally subjected to SDS-PAGE. The SDS-PAGE gel was fixed with 10% methanol and 10% acetic acid for 20 min, air-dried, and finally subjected to autoradiography.

**F-actin Cosedimentation Assays**—An actin cosedimentation assay was performed as described by the manufacturer using an actin binding protein spin-down assay kit from Cytoskeleton, Inc. Briefly, GST-recombinant proteins of Amot were incubated with 20 μg of freshly polymerized actin (F-actin) for 1 h at room temperature. After incubation, the protein plus F-actin solution was subjected to high-speed centrifugation (80,000 rpm, 1 h) to pellet F-actin and protein bound to F-actin. The pellet fraction was solubilized in SDS sample buffer (using the same volume as the supernatant fraction). Equivalent volumes of the pellet and supernatant fractions were analyzed by SDS-PAGE, followed by immunoblotting with anti-actin and anti-GST.

**RESULTS**

**Amot Family Members Are Substrates of the Hippo Core Kinase Complex**—Because the Hippo core kinase complex phosphorylates Yorkie in flies and YAP and TAZ in mammals via the consensus HXRxXS motif, we reasoned that it is possible to identify candidate substrates through identifying proteins with the same consensus motif. Because the signaling pathway is generally known to have a feedback mechanism, it is possible that the Hippo core kinase complex phosphorylates other components of the Hippo pathway besides YAP and TAZ. We visually examined the amino acid sequence of all known human components of the Hippo pathway. This analysis revealed that Amot and AmotL2 have a single HXRxXS motif, whereas AmotL1 has three such motifs (Fig. 1A). The first HXRxXS motif of AmotL1 is located at the corresponding region of the sole motif of Amot and AmotL2. This site (HVRSLS) is totally conserved, and the flanking sequences are highly conserved among the three Amot proteins (Fig. 1A, bottom panel). We therefore decided to focus on this site.

To test whether Amot family members are truly substrates of the Hippo pathway, we coexpressed HA-tagged Amot, AmotL1, and AmotL2 either with a vector control or constructs for expressing MST2, WW45, LATS2, and MOB1 (referred to as Hippo core kinases) in HEK293 cells. The cell lysates were resolved by phospho-tag gel followed by immunoblot analysis to detect HA-tagged Amot family proteins. The altered mobility and/or abundance are evidence of increased phosphorylation. As shown in Fig. 1B, in the presence of Hippo core kinases, the abundance of all three proteins was greatly increased. Furthermore, the mobility of AmotL1 and AmotL2 is noticeably retarded, suggesting that Hippo core kinases caused increased phosphorylation of all three proteins. To validate the site of phosphorylation as well as to detect phosphorylation of endogenous Amot proteins, we used peptides phosphorylated at the serine residues of the HXRxXS motif corresponding to the Amot and AmotL2 sequences to raise phospho-specific rabbit antibodies. To check for specificity of the phospho-Amot antibody, cell lysates were pretreated with λ-phosphatase under different conditions. As shown in Fig. 1C, phosphorylation of this site of the endogenous Amot detected by phospho-Amot antibody was increased in the presence of LATS2 and MOB1 (Fig. 1C, compare lanes 1 and 3) but disappeared when the lysates were treated with λ-phosphatase (lane 4). This indicates that the phospho-Amot antibody specifically recognizes phosphorylated Amot. Also of note is that the total amount of Amot was increased after being phosphorylated by LATS (Fig. 1C) and reduced when lysates were pretreated with λ-phosphatase, indicating that phosphorylation of Amot by LATS enhances the protein stability of Amot. Similarly, phosphorylation of the serine at HXRxXS of AmotL2 detected by phospho-AmotL2 antibody is also increased in the presence of the Hippo core kinases (data not shown).

To validate that LATS1/2 is indeed the kinase responsible for the phosphorylation of Amots endogenously, LATS1 and LATS2 were knocked down in HEK293 cells. Fig. 1D shows that when LATS1 and LATS2 were knocked down, phosphorylated Amot recognized by the specific phospho-Amot antibody was greatly reduced (Fig. 1D, top panel). In this experiment, YAP phosphorylation was also reduced, consistent with our knowledge that YAP is phosphorylated by LATS1 and LATS2. It is noteworthy that the amount of Amot increased in the presence of LATS kinase, indicating that phosphorylation of Amots by LATS1/2 stabilizes the proteins.

Because the site and flanking sequences are well conserved among Amot, AmotL1, and AmotL2, we tested whether the phospho-Amot antibody can also detect Hippo-mediated phosphorylation of AmotL1 and AmotL2. As shown in Fig. 2A, when coexpressed with Hippo core kinases, HA-tagged Amot (lane 4), AmotL1 (lane 12), and AmotL2 (lane 8) were robustly phosphorylated, as detected by the phospho-Amot-specific antibody. These results establish that, like Amot, the site of AmotL1 and AmotL2 is similarly phosphorylated by Hippo core kinases and that the phospho-Amot antibody can detect phosphorylated forms of all three Amot family members. Similarly, phospho-AmotL2 antibody could recognize all three phosphorylated Amot family members (data not shown). To further verify that the sole HXRxXS site of Amot and AmotL2 is indeed phosphorylated by Hippo core kinases, HXRxXS site mutants of Amot (S175A) and AmotL2 (S159A) were made. As shown in Fig. 2A, mutation of the HXRxXS sites of Amot (S175A) (lanes 5 and 6) and AmotL2 (S159A) (lanes 9 and 10) completely abolished the detection of the phosphorylated forms by the antibody.

To further determine whether the phosphorylation of Amot at serine 175 is mediated by LATS kinase itself, an in vitro kinase assay was performed. As shown in Fig. 2B, immunoprecipitated LATS2 kinase in vitro-phosphorylated fusion protein GST-Amot (1–409) but much less for GST-Amot (1–409)-S175A (compare lanes 5 and 6). The immunoprecipitated kinase-dead LATS2 (LATS2-KD) fails to in vitro-phosphorylate GST-Amot (1–409) (Fig. 2B, lane 8) and
GST-Amot (1–409)-S175A (lane 9) as compared with wild-type LATS2 (lanes 5 and 6). Moreover, AmotL2 phosphorylation of the HXRXXS site was totally dependent on the kinase activity of LATS2 because the phosphorylation of AmotL2, detected by phospho-AmotL2 antibody, and the phosphorylation of YAP (as a positive control) were not
detected when kinase-dead LATS2 mutants were used (Fig. 3A, lanes 3 and 4).

The Amot family has been shown to be activator of LATS kinases (20). As expected, AmotL2 promoted LATS2-mediated phosphorylation of YAP (Fig. 3B, lane 3), but when coexpressed, AmotL2-S159A did not promote the enhancement of YAP-Ser-127 phosphorylation by LATS2 (Fig. 3B, lane 4) as compared with coexpression of wild-type AmotL2 (lane 3).

Because NDR1 (STK38) and NDR2 (STK38L) work similarly to LATS kinase, we investigated whether knockdown of NDR1 (STK38) and NDR2 (STK38L), along with MST1 and MST2, affect the phosphorylation state of Amot. siRNA knockdown of STK38, STK38L, MST1, and MST2 in cells was not efficient, even though we tried different sources of siRNAs. Instead, we used a different approach. As shown in Fig. 3C, overexpression of STK38 or STK38L only marginally enhanced phosphorylation of endogenous Amot (Fig. 3C, lanes 2 and 8, top panels). When STK38 or STK38L were co-overexpressed with MST2, Amot phosphorylation was increased more significantly (Fig. 3C, lanes 3 and 9, top panels). To evaluate the role of LATS1/2 in the phosphorylation of endogenous Amot because of overexpression of STK38 or STK38L with MST2, LATS1/2 was knocked down in these cells. The results show that when LATS1/2 was knocked down, the Amot phosphorylation stim-
Phosphorylation by Hippo Core Kinases Inhibits the Actin-binding Activity of Amot—To examine the functional consequence of Hippo pathway-mediated phosphorylation of Amot proteins, we generated a non-phospho mutant (S175A) and phospho-mimic mutants (S175D and S175E) of Amot. Because Amot family members are key components and regulators of the junctional complex of tight epithelial cell layers (21), we examined the junctional localization of Amot, Amot-S175A, Amot-S175D, and Amot-S175E in a dense culture of MCF10A cells. As shown in Fig. 4A, Amot is enriched in the junctional complex, marked by an actin cytoskeleton with some background distribution in the cytoplasm. The junctional targeting of Amot-S175A was enhanced with concomitantly less cytoplasmic distribution (Fig. 4B). In marked contrast, Amot-S175D and Amot-S175E (data not shown) were distributed essentially in the cytoplasm, with no enrichment in the junctions with actin (Fig. 4C). Interestingly, in the sparse cells, colocalization of Amot and Amot-S175A with the actin cytoskeleton was seen (Fig. 4, D and E). Importantly, a robust localization of Amot-S175A with the actin cytoskeleton was observed (Fig. 4F). Amot-L1 and Amot-p80 also did not show colo-

FIGURE 3. LATS kinase activity is important for Amot phosphorylation and its effect on YAP phosphorylation. A, LATS kinase activity is required for the phosphorylation of AmotL2. HEK293 cells were transfected with FLAG-YAP and HA-AmotL2 either with a vector, HA-LATS2, or LATS2 kinase-dead mutants (HA-LATS2-KD-S872A and HA-LATS2-KD-T1041A). Transfected cells were harvested, and Western blotting was done with anti-phospho-AmotL2 antibody to detect phosphorylated AmotL2, anti-phospho-YAP to detect phosphorylated YAP-S127, anti-FLAG antibody to detect total YAP, anti-HA antibody to detect HA-LATS2 and mutants and HA-AmotL2, and anti-actin as the loading control. B, phosphorylation of AmotL2 at Ser-159 by LATS is required for the enhancement of YAP phosphorylation by LATS. HEK293 cells were transfected with the indicated combinations of constructs, and Western blotting was done using the antibodies mentioned in A to detect the indicated proteins. C, STK38 and STK38L do not directly phosphorylate Amot. Lysates of Myc-STK38, Myc-STK38L and FLAG-MST, Myc-STK38L, Myc-STK38L and FLAG-MST2 or a vector control expressing HEK293 cells with or without LATS1 and 2 knockdown were run on SDS-PAGE, and Western blotting was done using the indicated antibodies.
calization with actin (data not shown). These results suggest that Hippo phosphorylation of Amot may inhibit the association of Amot with actin.

To further validate this conclusion derived from studies of mutants, we coexpressed LATS2 with AmotL2 in dense MDCK cells. As shown in Fig. 5A, in cells with more coexpressed LATS2 (cell 1), AmotL2 was dissociated from the junction and redistributed in the cytoplasm (cell 1) as compared with junctional association in cells with less coexpression of LATS2 (cells 2 and 3). This result suggests that phosphorylation of Amots by LATS plays a role in the localization of Amot in the cells.

Endogenous Amot protein localization in the cells was also examined. In HEK293 cells, one of the few cell lines that express Amot endogenously, Amot was localized at the junction associated with actin (Fig. 5B). The Amot antibody was specific because it recognized only HA-tagged Amot but not HA-tagged AmotL1 or AmotL2 in addition to endogenous Amot (Fig. 5C, upper panel), whereas the HA antibody detected HA-tagged Amot, AmotL1, and AmotL2 (Fig. 5C, middle panel). Actin was used as a loading control (Fig. 5C, lower panel).

To further examine the localization of the phosphorylated Amot in the cells, we used MCF10A cells expressing HA-Amot. As shown in Fig. 6A, the phosphorylated antibody detected mainly the punctate staining and broad cytoplasmic staining. When LATS1 and LATS2 were knocked down in the cells, phospho-Amot staining is dramatically reduced (Fig. 6A, bottom row), indicating that the staining of phosphorylated Amot is specific and that the LATS1/2 is responsible for Amot phosphorylation. Because Amot is shown to be associated with the tight junction, we investigated whether Amot mutants might affect the localization of the tight junction protein. The results show that the phospho-mimic mutant of Amot, Amot-S175D, did not seem to affect the tight junction because the tight junction protein ZO-1 localization appeared normal (Fig. 6B).
Amots as Hippo Pathway Substrates

Amot Mutants Have Differential Effects on Cell Proliferation—Because the Hippo pathway regulates cell growth and proliferation, we decided first to determine whether Amot phosphorylation is regulated differently under different growth conditions. The results (Fig. 8A, left top panel) show that Amot was more extensively phosphorylated in dense cells but less in sparse cells. YAP and LATS1 were also phosphorylated more robustly in dense cells as compared with sparse cells, as expected. Phosphorylated LATS1 at Thr-1079 in dense cells increases the kinase activity of LATS1, which, in turn, may phosphorylate YAP and Amot. The total amount of Amot was also increased in dense cells as well, and this is consistent with the results shown in Fig. 1, showing that in the presence of LATS1/2, the total amount of Amot was increased but decreased when endogenous LATS1/2 was knocked down (Fig. 1D, second panel). Again, the results indicate that phosphorylation of Amot at Ser-175 plays a role in the stability of Amot. Knockdown of LATS1/2 in dense cells resulted in decreased phosphorylation of Amot and YAP (Fig. 8A, top right panel), indicating that LATS1/2 is responsible for the increased phosphorylation of Amot (in addition to YAP) in dense cells.

YAP/TAZ is an important downstream target of the Hippo pathway, and we have shown previously that Amot inhibits YAP activity by translocating YAP to the cytoplasm and inhibits YAP transcriptional activation of the target genes CTGF and Cyr61. Experiments were also conducted to investigate the effects of Amot and Amot-S175A, D, and E on YAP interaction, YAP localization, and YAP/TAZ target gene expression. As shown in Fig. 8, B–F, Amot-S175A interacted less with phospho-YAP as compared with Amot and Amot-S175D and E (mimic-phosphorylated Amot). Amot-S175D and E interacted a lot more with phospho-YAP compared with S175A and Amot (Fig. 8, B and C). Amot-S175A also inhibited less the expression of the YAP/TAZ target genes CTGF (Fig. 8D) and Cyr61 (E), and MCF10A-expressing Amot-S175A cells had less phosphorylated, cytoplasmically localized YAP as compared with Amot- and Amot-S175D- and E-expressing cells, which essentially eliminated nuclear YAP (F). Taken together, these results indicate that phosphorylation of Amot by LATS does play a role in regulating the activity of YAP/TAZ.

In addition, Amot and mutants have differential effects on cell proliferation. As shown by the colony formation assays in Fig. 9A, Amot, Amot-S175D, and Amot-S175E inhibited cell proliferation as compared with control parental cells, but Amot-S175A grew much better. Western blot analysis showed a comparable expression of Amot and its mutant in the cells (Fig. 9B). These results are consistent with the growth curves shown in Fig. 9C, where Amot-S175A cells grew significantly faster, whereas Amot, Amot-S175D, and -S175E cells grew slower than control cells. Taken together, these results imply that the Hippo pathway-mediated phosphorylation of Amot shifts Amot toward inhibiting cell proliferation, whereas the unphosphorylated form promotes proliferation, which may correlate with differential properties in association with actin filaments.

skeleton in sparse cells, whereas Amot-S175D did not show any colocalization with actin, in the context that Amot-S175A has increased targeting to the actin cytoskeleton-marked junction, one possibility is that the interaction with the actin cytoskeleton is the basis for junctional targeting of Amot. Actin cosedimentation experiments were used to detect the actin-binding activity of Amot. Fig. 7A reveals that GST-Amot (1–409) and GST-Amot (1–409)-S175A could bind directly with actin filaments (Fig. 7A, right panel) but not with Amot (1–409)-S175D and E. In the presence of actin filaments, Amot and Amot-S175A were primarily segregated into the pellet, whereas they were primarily present in the soluble fraction when the experiment was performed in the absence of actin filaments.

When full-length HA-Amot and mutants were expressed in HEK293 cells, actin was pulled down by HA-Amot and HA-Amot-S175A, with Amot-S175A retaining a greater amount of actin (Fig. 7B, top panel, compare lanes 2 and 3). However, HA-Amot-S175D and E failed to retain actin in their immunoprecipitates (Fig. 7B, lanes 4 and 5). Amot was also shown to be able to interact with actin endogenously (Fig. 7C). To map the actin-binding site of Amot, different fragments of Amot were fused to GST and were used to do actin-binding assays. As shown in Fig. 7D, fragments encompassing residues 1–100 and 1–150 did not interact with F-actin, but fragment 1–200 interacted with actin, indicating that residues 150–200 of Amot were important for actin binding. Combined with the results shown in Fig. 7, A–C, showing that Amot-S175D and E did not interact with F-actin, serine 175 of Amot is an important residue regulating actin binding. Taken together, the results indicate that the phosphorylation of Amot by LATS has a negative effect on Amot interaction with actin.

FIGURE 6. Punctate staining of phosphorylated Amot in MCF10A cells. A, localization of phospho-Amot in MCF10A cells overexpressing HA-Amot. The cells were either transfected with control (ctrl) siRNA or LATS1 and 2 siRNA. After 72 h, the cells were stained with anti-HA (red) for Amot and anti-phospho-Amot for phosphorylated Amot (green). Scale bar = 10 μm. B, Amot and mutant do not seem to affect the distribution of tight junction protein ZO-1.
DISCUSSION

This study aimed to investigate substrates of LATS kinase other than the Hippo pathway downstream target YAP/TAZ. The Hippo pathway is a newly discovered signaling pathway regulating organ size through cell growth, cell proliferation, and apoptosis. There is mounting evidence showing that the Hippo pathway is an important pathway. Mutations or deregulation of the components of the pathway are gradually found in cancers. The main components of the pathway are the core kinase complex, which consists of kinases MST1/2 and LATS1/2 working with adaptor proteins WW45 and MOB. Until now, the main downstream substrate of the core kinase LATS1/2 was YAP/TAZ. When the Hippo pathway is activated by still not very clearly defined upstream factors, LATS phosphorylates and inactivates YAP/TAZ, inhibiting their transcriptional activity in the nucleus. After being phosphorylated by LATS, YAP and TAZ are translocated to the cytoplasm, and, hence, inhibition of cell growth and proliferation are achieved. We believe that there are LATS substrates other than YAP/TAZ and that this additional regulation of components of the pathway by LATS may add another layer of regulation to the pathway. By inspecting the sequences of known components of

FIGURE 7. Actin-binding activity of Amot. A, Amot and Amot-S175A bind directly to F-actin. An actin cosedimentation assay was used to examine the binding of Amot and Amot-S175A to F-actin. Recombinant GST-Amot (1–409) and GST-Amot-S175A (1–409) were incubated with or without F-actin and subjected to high-speed centrifugation. Soluble (S) and pellet (P) fractions were resolved by SDS-PAGE and immunoblotted with anti-GST antibody to detect GST-Amot (1–409) and mutants (right panel). GST-actinin and GST were used as positive and negative controls (left panel), respectively. B, full-length Amot and Amot-S175A, but not Amot-S175D and E, associate with actin in HEK293 cells. Lysates from HEK293 cells transfected with HA-Amot, HA-Amot-S175A, HA-Amot-S175D, HA-Amot-S175E, and a vector control were immunoprecipitated (IP) with anti-HA and probed with anti-actin and anti-HA. C, Amot associated with actin endogenously. Lysates from HEK293 cells were immunoprecipitated either with anti-Amot or IgG control and probed with anti-actin and anti-Amot. D, mapping of the actin-binding site of Amot. Left panel, schematic showing different regions of Amot that fused with GST and their binding abilities to F-actin. Right panel, the actin cosedimentation assay described in A was used to determine the region of Amot that is important for associated with F-actin.
Amots as Hippo Pathway Substrates

the Hippo pathway, we found that angiomotin family members are potential substrates because there are conserved LATS sites in their sequences. Through careful investigation in this work, we found that Amots are indeed substrates of LATS kinase. Phosphorylation of Amot by LATS affects the activity of Amot, especially its actin-binding activity as well as its functions on cell proliferation. Phosphorylated Amot negatively regulates cell proliferation through its inability to interact with actin as well as its ability to inhibit YAP. One important finding is that Amot was found to be more stable when it was phosphorylated by LATS. Phosphorylated Amot, in turn, interacts more with YAP and inhibits the transcriptional activity of YAP. This indicates that LATS regulates multiple components of the Hippo pathway to regulate cell growth and proliferation. There is a possibility that a change in the cell proliferation rate may result from altered actin dynamics (and cytokinesis) during the cell cycle. Amot may regulate cell proliferation through its interaction with...
Further study is needed to address this issue. During the preparation of this manuscript, there were three papers that also show that Amot is phosphorylated by LATS (22–24). These results collectively suggest the following working model. Amot family members exist dynamically between the unphosphorylated and phosphorylated forms regulated by Hippo core kinases. Unphosphorylated Amot has an increased interaction with the actin cytoskeleton and is therefore targeted to the junction when the actin cytoskeleton is assembled at the junction in epithelial cells when cells are attached to each other. This dynamic association with actin has a positive role in cell proliferation. When not phosphorylated, Amot is able to interact with actin filament and plays a positive role in cell proliferation. Upon phosphorylation by Hippo kinases, phosphorylated Amot dissociates from actin filament and has increased interaction with YAP and TAZ, resulting in their enhanced cytoplasmic retention. At the same time, phosphorylated Amot provides a feedback to enhance YAP/TAZ phosphorylation by the Hippo pathway. These together result in inhibition of cell proliferation.
TAZ in the cytoplasm to prevent their nuclear targeting and, therefore, to inhibit cell proliferation (Fig. 9D). Because Amot family members are not present in flies, the mammalian system may have evolved this novel regulation to accommodate more complicated cellular and physiological needs to regulate organ size control.

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