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Hippo Pathway-independent Restriction of TAZ and YAP by Angiomotin*

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The Hippo pathway restricts the activity of transcriptional co-activators TAZ and YAP by phosphorylating them for cytoplasmic sequestration or degradation. In this report, we describe an independent mechanism for the cell to restrict the activity of TAZ and YAP through interaction with angiomotin (Amot) and angiomotin-like 1 (AmotL1). Amot and AmotL1 were robustly co-immunoprecipitated with FLAG-tagged TAZ, and their interaction is dependent on the WW domain of TAZ and the PP*XY* motif in the N terminus of Amot. Amot and AmotL1 also interact with YAP via the first WW domain of YAP. Overexpression of Amot and AmotL1 caused cytoplasmic retention of TAZ and suppressed its transcriptional outcome such as the expression of *CTGF* and *Cyr61*. Hippo refractory TAZ mutant (S89A) is also negatively regulated by Amot and AmotL1. HEK293 cells express the highest level of Amot and AmotL1 among nine cell lines examined, and silencing the expression of endogenous Amot increased the expression of *CTGF* and *Cyr61* either at basal levels or upon overexpression of exogenous S89A. These results reveal a novel mechanism to restrict the activity of TAZ and YAP through physical interaction with Amot and AmotL1.

The Hippo pathway is emerging as an important signaling pathway to regulate organ size *in vivo* and cell contact inhibition *in vitro* through control of cell proliferation and apoptosis. The Hippo pathway was originally defined in fly *Drosophila melanogaster* and is structurally, functionally, and mechanistically conserved in mammals (1–5). In the mammals, the upstream regulators such as NF2-Merlin activate the core kinase machinery composed of Mst1/2, WW45, LATS1/2, and Mob1, leading to inactivation of transcriptional co-activators YAP (Yes-associated protein) and TAZ (transcriptional co-activator with PDZ-binding motif). YAP and TAZ are homologous to each other, and they represent the mammalian counterpart of fly Yorkie. YAP and TAZ contain an N-terminal region responsible for interaction with TEAD1–4 transcriptional factors followed by one (for TAZ) or two (for YAP) WW domains that enable them to interact with proteins containing PP*XY* motifs (4, 6–11). The WW

domain has been shown to interact with several PP*XY* motif-containing proteins such as Wbp2 (12), p73 (13), Runx2 (14–16), and Smads (17). The C-terminal region of TAZ and YAP contains a transcriptional activation domain able to stimulate the transcriptional outcome of the cognate transcriptional factors. The C terminus of YAP and TAZ also contains a PDZ-binding motif enabling them to interact with PDZ domain-containing proteins. Among the many transcriptional factors that have been reported to interact with YAP and TAZ, TEAD1, -2, -3, and -4 (and the fly Scalloped) are mostly involved in regulating the transcriptional outcome to govern cell proliferation and apoptosis. Many potential downstream target genes for TAZ-YAP-TEADs have been revealed by microarrays (11, 18), and *CTGF* and *Axl* are experimentally shown to be direct target genes (12, 19).

The Hippo pathway represents a major regulatory control to restrict the activity of YAP and TAZ by affecting their subcellular distribution and stability (10, 11, 18, 20–23). Upon activation, the protein kinase complex LATS1/2-Mob1 phosphorylates multiple Ser residues within the H*X*R*XX*S motif of YAP and TAZ. When Ser[89] of TAZ and Ser[127] of YAP are phosphorylated, YAP and TAZ are sequestered in the cytoplasm by interaction with 14-3-3 proteins. Furthermore, phosphorylation of Ser[314] of TAZ and Ser[381] of YAP primes them for subsequent phosphorylation by casein kinase 1 followed by ubiquitination and proteasomal degradation (21, 22). Whether there exist other regulatory pathways to restrict the activity of TAZ and YAP is not known.

Angiomotin (Amot)[2] was initially identified as a binding protein of angiostatin to regulate endothelial cell migration (24) and is expressed as two isoforms (p130 and p80) with p130 containing an N-terminal extension (25). Two proteins homologous to Amot are termed AmotL1 and AmotL2 (25–27). Amot family members have been shown to interact with the actin cytoskeleton (25) and be part of the cell junctional complex (25–28). An extensive proteomic study has shown that Amot interacts with Rich1, a Cdc42 RhoGAP, as well as the Patj-Pals junctional complex to coordinate epithelial cell polarity (28). In this study, we describe our results showing that Amot and AmotL1 are novel regulators that interact with TAZ and YAP, leading to their cytoplasmic retention and inhibition of their transcriptional outcome and oncogenic property. Because Hippo refractory mutant S89A of TAZ is also

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[2] The abbreviations used are: Amot, angiomotin; co-IP, co-immunoprecipitation; ITC, isothermal titration calorimetry.

interacting with and is functionally inhibited by Amot and AmotL1, Amot and AmotL1 are able to restrict TAZ and YAP in a Hippo pathway-independent manner.

EXPERIMENTAL PROCEDURES

Cell Lines—All the cell lines were purchased from the American Type Culture Collection and maintained in the recommended media except for MCF10A, which was described previously (10, 12, 29, 30).

Antibodies—All the antibodies used were described previously (10, 12, 29, 30) except antibodies for Amot and AmotL1, which were from Abnova and Sigma, respectively, and anti-HA agarose conjugate, which was from Sigma.

Immunofluorescence, Anchorage-independent Growth in Soft Agar, RNA Isolation and Real-time PCR, Retrovirus Generation and Infection, and Immunoprecipitation—These were described previously (10, 12, 29, 30).

Plasmids—FLAG-TAZ(WT), FLAG-TAZ-S89A, FLAG-TAZ-WWm, FLAG-YAP-WT, FLAG-YAP-S127A, FLAG-YAP-WW1m, FLAG-YAP-WW2m, and FLAG-YAP-WW1+2m were previously described (10, 12, 29, 30). Alanine was introduced to the Tyr residue of the PP*XY* motif of Amot-PP*XY* mutants by PCR. Amot and AmotL1 full-length cDNA were purchased from OriGene. HA epitopes were tagged at the N termini of Amot and AmotL1 and cloned into the pCIneo expression vector (Promega).

siRNA—Control (non-target), Amot, and AmotL1 smart pool siRNAs were purchased from Dharmacon. 10 μl of 100 μM siRNA was used to transfect 293 cells in a 6-well plate using DharmaFECT 4 (Thermo Scientific) as transfection reagent according to the company's recommended protocol. Cells were harvested after 72 h for RT-PCR to measure TAZ target gene expression and for Western blotting to measure knockdown efficiency.

Isothermal Titration Calorimetry (ITC)—The proteins used for ITC are YAP-WW1+2 (amino acids 171–267), YAP-WW1 (amino acids 171–208), YAP-WW2 (amino acids 230–267), TAZ-WW (amino acids 124–157), and Amot (amino acids 8–317). All the proteins were expressed as His tag fusions in *Escherichia coli* BL21 (DE3) cells. After reaching an optimal density, the cells were induced with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside and were grown overnight at room temperature. In the case of the Amot fragment, the cells were grown at 15 °C for 5 h after induction. After cell lysis, the proteins were affinity-purified using a nickel-chelating column and were further purified using a Superdex 75 gel filtration column. The purified proteins were dialyzed in 20 mM Tris, pH 8.0, 100 mM NaCl, 2 mM β-mercaptoethanol. The measurement was done at 25 °C using $\sim$230 μM WW domain and $\sim$18 μM Amot fragment.

RESULTS

Amot and AmotL1 as Interacting Proteins of TAZ and YAP—In our large scale co-immunoprecipitation (co-IP) experiments, Amot and AmotL1 were preferentially co-recovered with TAZ and S89A but under-represented in the co-IP of WWm, a mutant TAZ with its WW domain mutated (12). This indicates that the WW domain of TAZ is important for

interaction with Amot and AmotL1. To validate the interaction, we have performed analytic co-IP experiments using HEK293 cells expressing FLAG-TAZ (Fig. 1*A*). Cell lysates were immunoprecipitated with anti-FLAG antibodies to test the co-recovery of endogenous Amot and AmotL1. As shown, endogenous Amot (*left panel*) and AmotL1 (*right panel*) were efficiently co-immunoprecipitated with FLAG-TAZ (*lane 4*). To evaluate the importance of the Hippo pathway and WW domain of TAZ in mediating the interaction, we compared the interaction of S89A and WWm with wild-type TAZ. Lysates derived from cells transfected with FLAG-TAZ, -S89A, and -WWm were immunoprecipitated, and the co-recovery of endogenous Amot was detected (Fig. 1*B*). As shown, Amot was co-recovered with TAZ and S89A at high efficiencies (*lanes 6* and *7*), whereas WWm failed to interact with Amot (*lane 8*), suggesting that Amot interacts with TAZ in a manner that is dependent on the WW domain of TAZ regardless of whether Ser[89] is subjected to Hippo regulation. YAP is homologous to TAZ and is also a downstream target inhibited by the Hippo pathway, and its Ser[127] residue is the primary site for Hippo-mediated phosphorylation and sequestration by 14-3-3 proteins. As compared with TAZ having one WW domain, YAP has two WW domains. We therefore also tested the interaction of Amot with YAP (Fig. 1*C*). HEK293 cells were transfected to express FLAG-YAP, -WW1m (mutation of the first WW domain), -WW2m (mutation of the second WW domain), and -WW1+2m (mutation of both WW domains). As shown in Fig. 1*C*, HA-Amot was co-immunoprecipitated with FLAG-YAP and -WW2m (*lanes 7* and *9*, respectively), but not with WW1m or WW1+2m (*lanes 8* and *10*, respectively), suggesting that Amot interacts with YAP in a manner that is dependent on the first but not the second WW domain of YAP. We also tested the interaction of endogenous AmotL1 with the various mutants of TAZ and YAP (Fig. 1*D*). AmotL1 interacted with TAZ (*lane 2*), S89A (*lane 3*), YAP (*lane 5*), S127A (*lane 6*), and YAP-WW2m (*lane 8*) but not TAZ-WWm (*lane 4*), YAP-WW1m (*lane 7*), or YAP-WW1+2m (*lane 9*), suggesting that the WW domain of TAZ and the first but not the second WW domain of YAP are similarly important for interaction with AmotL1. Amot and AmotL1 contain two PP*XY* motifs in the N-terminal region (Amot: [239]PPEY[242] and [284]PPEY[287]; AmotL1: [310]PPEY[313] and [367]PPEY[370]) with the first PP*XY* motif also being conserved in AmotL2 ([210]PPQY[213]). Because PP*XY* motifs are preferred motifs for interacting with WW domains (7, 8), it is possible that interaction of Amot and AmotL1 with TAZ and YAP is mediated by the PP*XY* motifs of Amot and AmotL1 and the WW domain of TAZ and YAP. Interestingly, Amot is expressed in two isoforms (p130 and p80) with the smaller p80 isoform lacking the PP*XY* motif-containing N-terminal 409-residue region. When FLAG-TAZ was immunoprecipitated from transfected HEK293 cells, the endogenous p130 but not the p80 of Amot was co-recovered (Fig. 1*A*, *left panel*), supporting the role of PP*XY* motifs of Amot in mediating interaction with the WW domain of TAZ. We also generated mutants of Amot having the first (PP*XY*1m), the second (PP*XY*2m), or both (PP*XY*1+2m) motifs mutated. HA-tagged Amot and mutants were expressed in
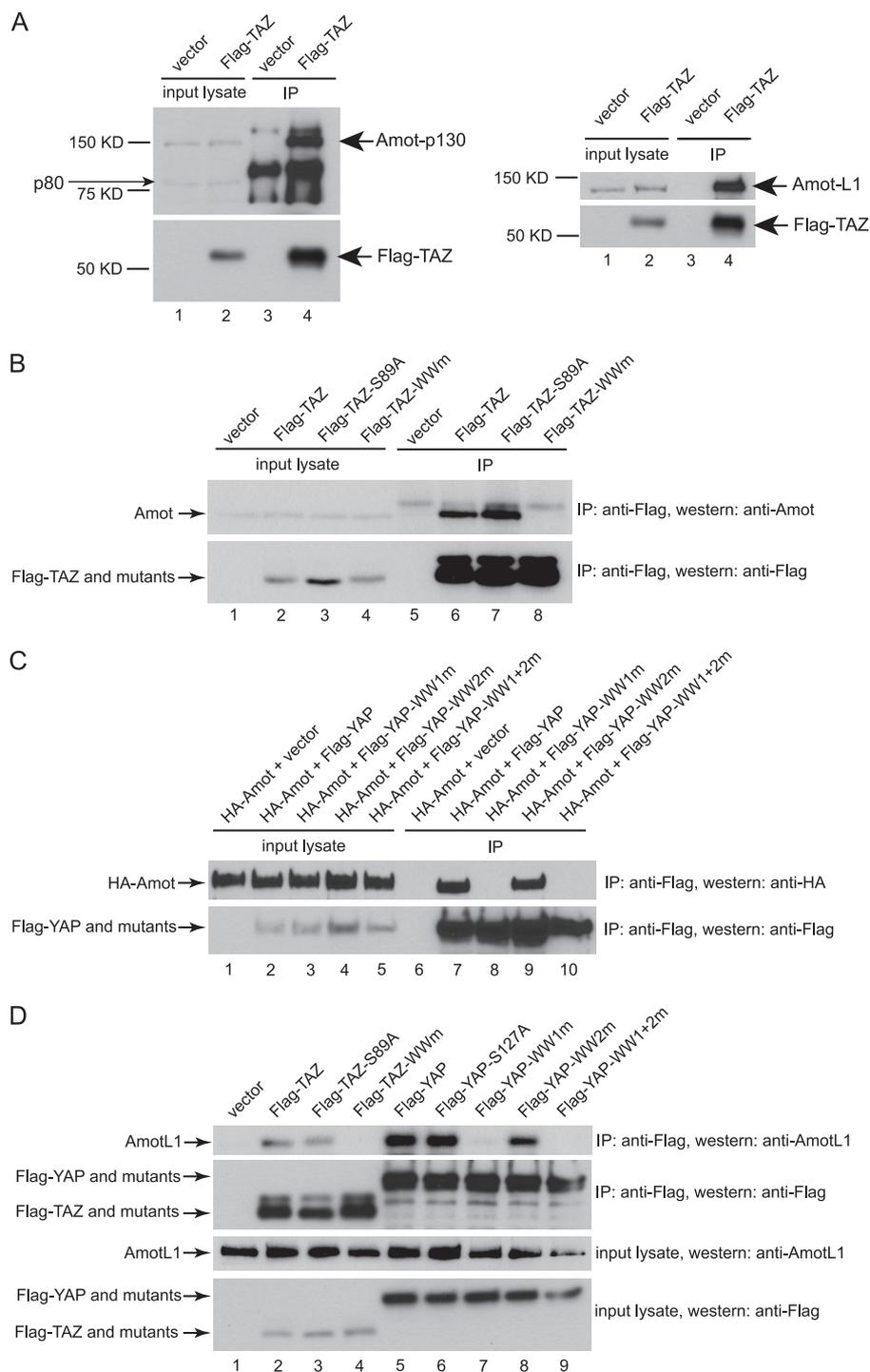
FIGURE 1. **Interaction of TAZ and YAP with Amot and AmotL1 is dependent of the WW domain and the PPXY.** *A*, lysates derived from HEK293 cells transfected with vector or FLAG-TAZ expression construct were immunoprecipitated with FLAG antibodies. The precipitates and lysates were analyzed by immunoblot to detect FLAG-TAZ and co-precipitated Amot (*left panel*) and AmotL1 (*right panel*). The p130 and p80 forms of Amot were indicated. *B*, lysates derived from HEK293 cells transfected with the indicated expression constructs were immunoprecipitated with FLAG antibodies. The precipitates and ly-sates were analyzed by immunoblot to detect FLAG-tagged TAZ proteins (*lower panel*) and co-precipitated endogenous Amot (*top panel*). *C*, lysates derived from HEK293 cells transfected with the indicated expression constructs were immunoprecipitated with FLAG antibodies. The precipitates and lysates were analyzed by immunoblot to detect FLAG-tagged YAP proteins (*lower panel*) and co-precipitated HA-Amot (*top panel*). *D*, lysates derived from HEK293 cells transfected with the indicated expression constructs were immunoprecipitated with FLAG antibodies. The precipitates were analyzed by immunoblot to detect FLAG-tagged proteins (*second panel*) and co-precipitated endogenous AmotL1 (*top panel*). The input lysates were analyzed for detection of AmotL1 (*third panel*) and FLAG-tagged protein (*bottom panel*). *E*, lysates derived from cells expressing HA-Amot and its indicated mutants were immunoprecipitated with anti-HA antibodies. The precipitates (*upper panels*) and lysates (*lower panels*) were processed for immunoblot to detect HA-Amot and co-recovered endogenous TAZ/YAP. *F–H*, direct interaction of PP*X*Y motif-containing Amot fragment with the YAP-WW1+2 domain. *F*, the *top panel* is the raw heat re-sponse obtained after injection of YAP-WW1+2 domain into ITC cell containing the N-terminal fragment of Amot. The *bottom panel* reflects the integrated peak areas normalized to moles of YAP-WW1+2, and the *solid line* is the least-squares fit to the binding isotherm. The affinity is 9 ± 1 μm. *G*, no significant heat response and binding when YAP-WW1+2m was injected. In this mutant, the tryptophan in the binding site of both WW domains is mutated to ala-nine. *H*, the affinity between the N-terminal fragment of Amot and various WW domains are tabulated. *aa*, amino acids.
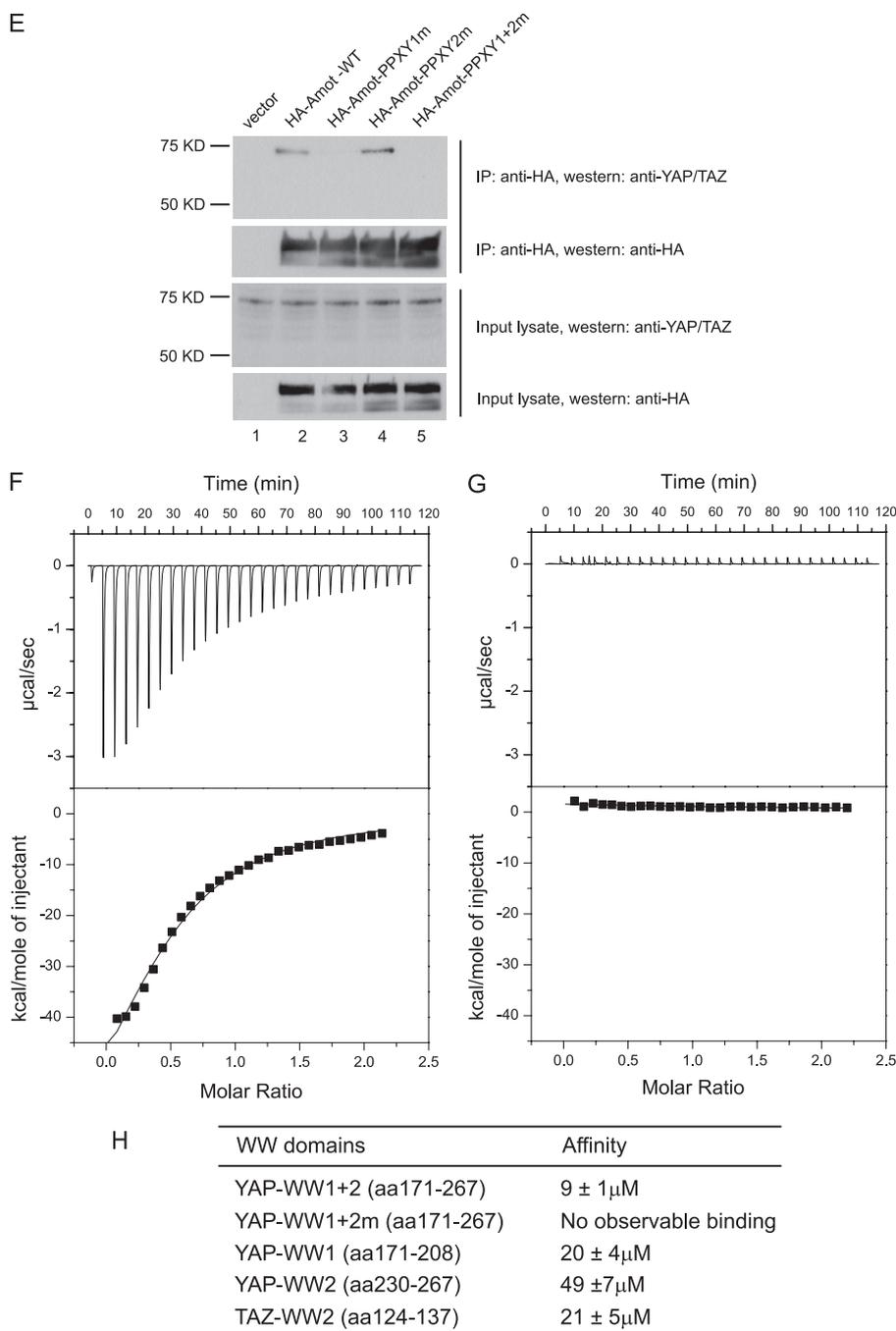
E

IP: anti-HA, western: anti-YAP/TAZ

IP: anti-HA, western: anti-HA

Input lysate, western: anti-YAP/TAZ

Input lysate, western: anti-HA

F

G

H

WW domains	Affinity
YAP-WW1+2 (aa171-267)	$9 \pm 1\mu M$
YAP-WW1+2m (aa171-267)	No observable binding
YAP-WW1 (aa171-208)	$20 \pm 4\mu M$
YAP-WW2 (aa230-267)	$49 \pm 7\mu M$
TAZ-WW2 (aa124-137)	$21 \pm 5\mu M$

FIGURE 1—*continued*

HEK293 cells and tested for their ability to co-recover endogenous YAP and TAZ (Fig. 1*E*). Endogenous YAP but not TAZ was detected by the antibodies that react with both YAP and TAZ (*lower panels* for input lysate). Importantly, YAP was co-recovered with HA-Amot (*lane 2*) and Amot-PP*X*Y2m (*lane 4*) but not Amot-PP*X*Y1m (*lane 3*) or Amot-PP*X*Y1–2m (*lane 5*) (*upper panel*), suggesting that the first but not the second PP*X*Y motif of Amot is important for interacting with endogenous YAP. Because the first but not the second motif is also conserved in AmotL2, it is likely that, like Amot and AmotL1, AmotL2 will also interact with TAZ and YAP. These results, taken together, suggest that the first PP*X*Y motif of Amot (and likely the cor-responding motif of AmotL1 and AmotL2) is responsible for interacting with the WW domain of TAZ and the first WW domain of YAP. In support of this notion, *in vitro* interaction assay by the ITC method using the PP*X*Y motif-containing region (residues 8–317) of Amot and recombinant fragment of the first and second WW domain of YAP (residues 171–267) revealed a direct and strong interaction of the Amot fragment with the WW domains (Fig. 1*F*). The interaction was completely abolished by mutation of the WW domains (Fig. 1*G*). Using isolated first and second WW domains, it was observed that first WW domain of YAP has a significantly stronger interaction with the Amot fragment (Fig. 1*H*).

Amot Negatively Regulates the Function of TAZ by Causing Its Cytoplasmic Sequestration—To investigate the functional consequence of TAZ interaction with Amot and AmotL1, we have first examined the distribution of TAZ-S89A in response to co-expression of Amot as distribution of TAZ and YAP between the cytoplasm and the nucleus is a major regulatory event executed by the Hippo pathway to restrict the transcriptional co-activator activity. The distribution of TAZ and YAP regulated by the Hippo pathway is also evident in response to cell density (10, 20, 23, 31). In sparse cells, TAZ and YAP are preferentially distributed in the nucleus, whereas in dense cell culture, when Hippo pathway is activated, TAZ and YAP are restricted more to the cytoplasm. FLAG-TAZ-S89A expressed in sparse cells was primarily detected in the nucleus (10, 11). When expressed in transfected cells, Amot was predominantly in the cytoplasm with some dotty structures and essentially excluded from the nucleus (Fig. 2*A*). In cells without co-expression of HA-Amot, FLAG-TAZ-S89A was essentially in the nucleus (*cell 1*). When HA-Amot was expressed moderately, some FLAG-TAZ-S89A was seen to be shifted to the cytoplasm (*cell 2*). FLAG-TAZ was mostly shifted to the cytoplasm when HA-Amot was expressed at high levels (*cells 3* and *4*). Similar results were obtained when TAZ, YAP, and S127A were co-expressed with Amot or AmotL1 (data not shown). These results suggest that Amot is able to restrict TAZ and YAP as well as their Hippo refractory mutants by sequestering them in the cytoplasm in a manner that is independent of the Hippo pathway. To further validate this, we have examined the expression of TAZ target genes such as *CTGF* and *Cyr61*. *CTGF* is a well defined target gene of TAZ and YAP, and its expression is a functional outcome of interaction of TAZ-YAP with TEAD1–4 as the promoter region of *CTGF* gene contains several TEAD-binding elements (12, 18, 23, 31). *Cyr61* was shown to be up-regulated by TAZ and YAP in several microarray studies (11, 18). As shown in Fig. 2, *B* and *C*, MCF7 cells were transfected with vector control (*columns 1–4*) or construct to express S89A (*columns 5–8*) alone (*columns 1* and *5*) or together with Amot (*columns 2* and *6*), AmotL1 (*columns 3* and *7*), or both Amot and AmotL1 (*columns 4* and *8*). The expression of endogenous *CTGF* and *Cyr61* genes was robustly increased by exogenous TAZ-S89A (*column 5*). When Amot and AmotL1 were co-expressed with S89A, enhanced expression of *CTGF* and *Cyr61* was compromised (*column 8*), suggesting that Amot and AmotL1 can negatively regulate the functional outcome of TAZ-S89A. Amot or AmotL1 alone had noticeable inhibition of S89A-induced expression of *CTGF* (Fig. 2*B*, *columns 6* and *7*, respectively). However, Amot and AmotL1 alone had little impact on S89A-induced expression of *Cyr61* (Fig. 2*C*, *columns 6* and *7*, respectively). These results suggest that in MCF7 cells, Amot and AmotL1 have additive and/or synergistic effect on inhibiting S89A-induced expression of endogenous *CTGF* and *Cyr61* genes. The expression of the exogenous S89A, Amot, and/or AmotL1 in these cells was validated by immunoblot analysis (Fig. 2*D*). We and others have shown that TAZ-S89A and YAP-S127A possess potent oncogenic property in NIH3T3 and MCF10A cells in conferring anchorage-independent cell growth in soft agar (10, 22, 23). This oncogenic property of S89A and S127A in NIH3T3 cells was also suppressed when Amot was co-expressed (Fig. 2, *E* and *F*). These results, taken together, suggest that Amot and AmotL1 can negatively regulate the functional outcome of TAZ-S89A, most likely through Hippo pathway-independent sequestration in the cytoplasm.

HEK293 Cells Have High Levels of Amot and AmotL1 Expression, and Knockdown of Amot but Not AmotL1 Enhances Expression of Endogenous CTGF and Cyr61 to Levels That Are Induced by S89A—We have recovered Amot and AmotL1 by large scale co-IP using HEK293 cells transiently expressing TAZ and S89A. To evaluate the influence of endogenous Amot and/or AmotL1 on the functional outcome of TAZ, we have examined the expression of Amot and AmotL1 in nine different human cell lines (Fig. 3*A*). As shown, HEK293 (*lane 9*) has the highest level of expression of Amot and AmotL1 among these cell lines. All other eight cell lines express low or undetectable levels of Amot and AmotL1 (*lanes 1–8*). We therefore used HEK293 cells to investigate the role of endogenous Amot and AmotL1. We first validated that the expression of endogenous Amot and AmotL1 was efficiently silenced by transfection of specific siRNA as the protein levels of Amot and AmotL1 were significantly reduced in cells transfected with their specific siRNA (Fig. 3*B*). We next examined the impact of knockdown of Amot, AmotL1, or both on the expression of TAZ target gene *CTGF* (Fig. 3*C*) and *Cyr61* (Fig. 3*D*). When Amot (*column 2*) but not AmotL1 (*column 3*) was silenced, the expression of *CTGF* and *Cyr61* was significantly enhanced. The levels of enhancement are almost comparable with those when the cells were expressing exogenous S89A (*column 5*). Interestingly, the enhanced expression of *CTGF* and *Cyr61* due to exogenous S89A can be further increased when endogenous Amot (*column 6*) but not AmotL1 (*column 7*) was suppressed by its siRNA. Therefore, endogenous Amot but not AmotL1 in HEK293 cells negatively regulates the expression of TAZ-YAP target genes *CTGF* and *Cyr61*, and this inhibition is evident both for endogenous YAP (as YAP but not TAZ was detected in this cell line as shown in Fig. 1*D*) as well as when exogenous S89A was expressed. These results obtained from this loss-of-function approach further support the notion that Amot can negatively regulate the function of TAZ and YAP.

DISCUSSION

TAZ and YAP function as transcriptional co-factors for many transcriptional factors such as TEAD1–4 (9–11, 18, 20, 23), p73 (13), TTF1 (32), Runx2 (14–16), TBX5 (33), peroxisome proliferator-activated receptor γ (PPARγ) (14), Pax3 (34), Smad2/3 (17), MyoD (14), and Glis3 (35). TEAD1–4 are the major transcriptional factors involved in the TAZ-YAP ability to regulate cell proliferation and apoptosis (1–3). As such, their availability and subcellular localization are tightly controlled to govern their functionality. The major known regulatory mechanism for TAZ and YAP is via the recently defined Hippo pathway (1, 3, 4). Upon activation, the core components of the Hippo pathway phosphorylate TAZ and YAP at multiple sites harboring the H*X*R*XX*S motif. When phosphorylated, phospho-Ser[89] of TAZ and phospho-Ser[127]
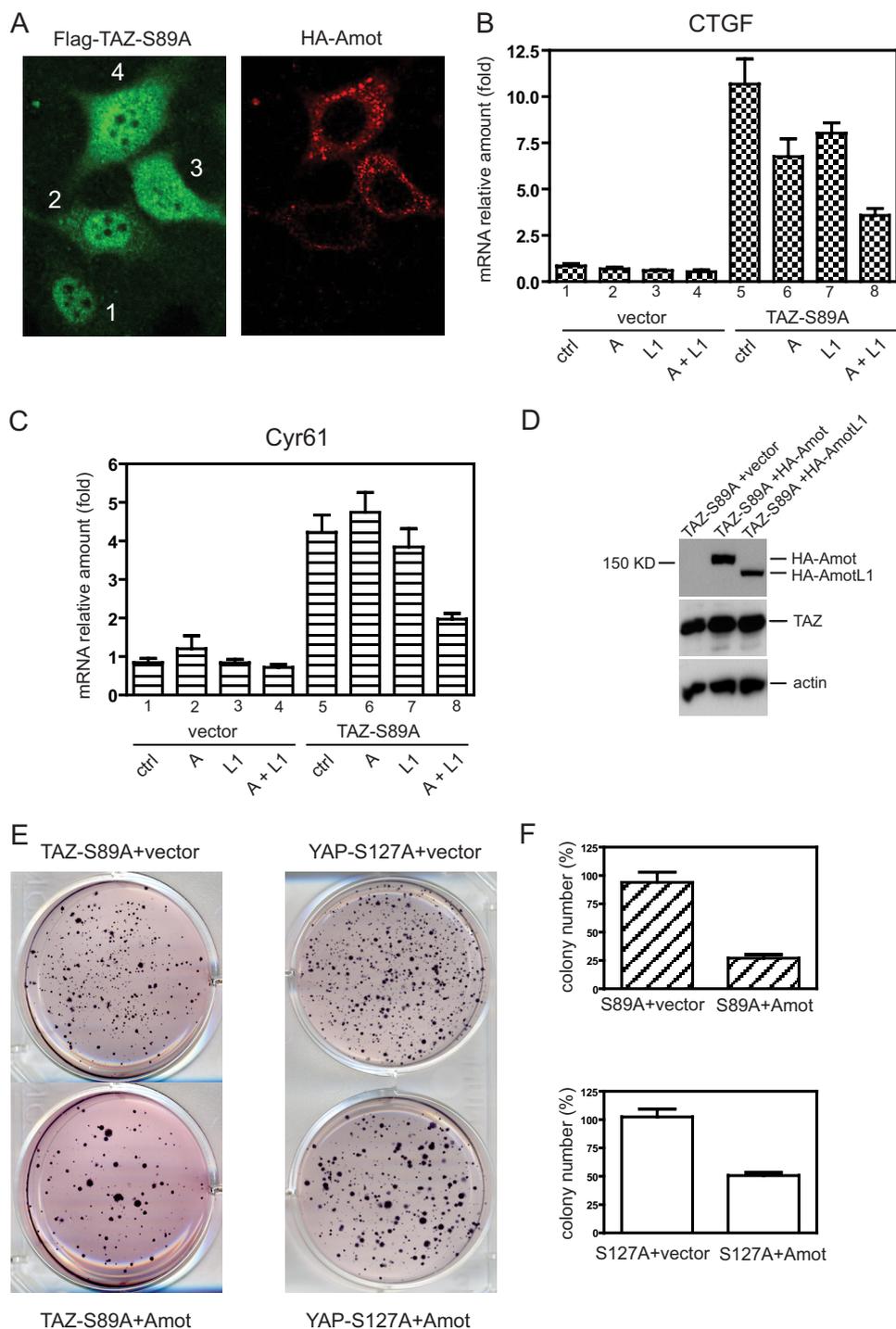
FIGURE 2. **Amot and AmotL1 inhibit TAZ transcriptional outcome and oncogenic property in promoting anchorage-independent growth.** *A*, MCF7 cells were co-transfected with FLAG-TAZ-S89A and HA-Amot. Cells were then processed to detect the expressed proteins using mouse anti-FLAG and rabbit anti-HA antibodies followed by secondary antibodies (*green* and *red* for FLAG and HA tag, respectively). FLAG-TAZ-S89A, expressed alone, is essentially in the nucleus (*cell 1*). When Amot was expressed at low moderate levels, some S89A was shifted to the cytoplasm (*cell 2*), and S89A was largely detected in the cytoplasm when Amot was expressed at high levels (*cells 3* and *4*). *B*, the mRNA levels of endogenous CTGF gene were measured by real-time PCR in cells transfected with vector (*columns 1–4*) or TAZ-89A-expressing construct (*columns 5–8*) together with the expression vectors indicated at the *bottom* (*ctrl*, vector control; *A*, Amot coding cDNA; *L1*, AmotL1 coding cDNA; *A+L1*, Amot and AmotL1 coding cDNAs together). The levels of CTGF mRNA were normalized to that detected in *column 1*, which was arbitrarily set at 1. *C*, the mRNA levels of Cyr61 were measured in those cells described in *panel B*. The Cyr61 mRNA levels were normalized to that detected in *column 1*, which was arbitrarily set at 1. *D*, the expression of S89A, Amot, or AmotL1 was examined by immunoblot. *E*, NIH3T3 cells were transduced to express TAZ-S89A (*left panel*) or YAP-S127A (*right panel*) along with vector (*upper panels*) or with Amot-expressing construct (*bottom panels*). Cells were grown in soft agar, and colonies were stained and photographed. *F*, the quantitative results derived from three independent experiments similar to *D* were shown. *Error bars* in *panels B*, *C*, and *F* indicate S.E.
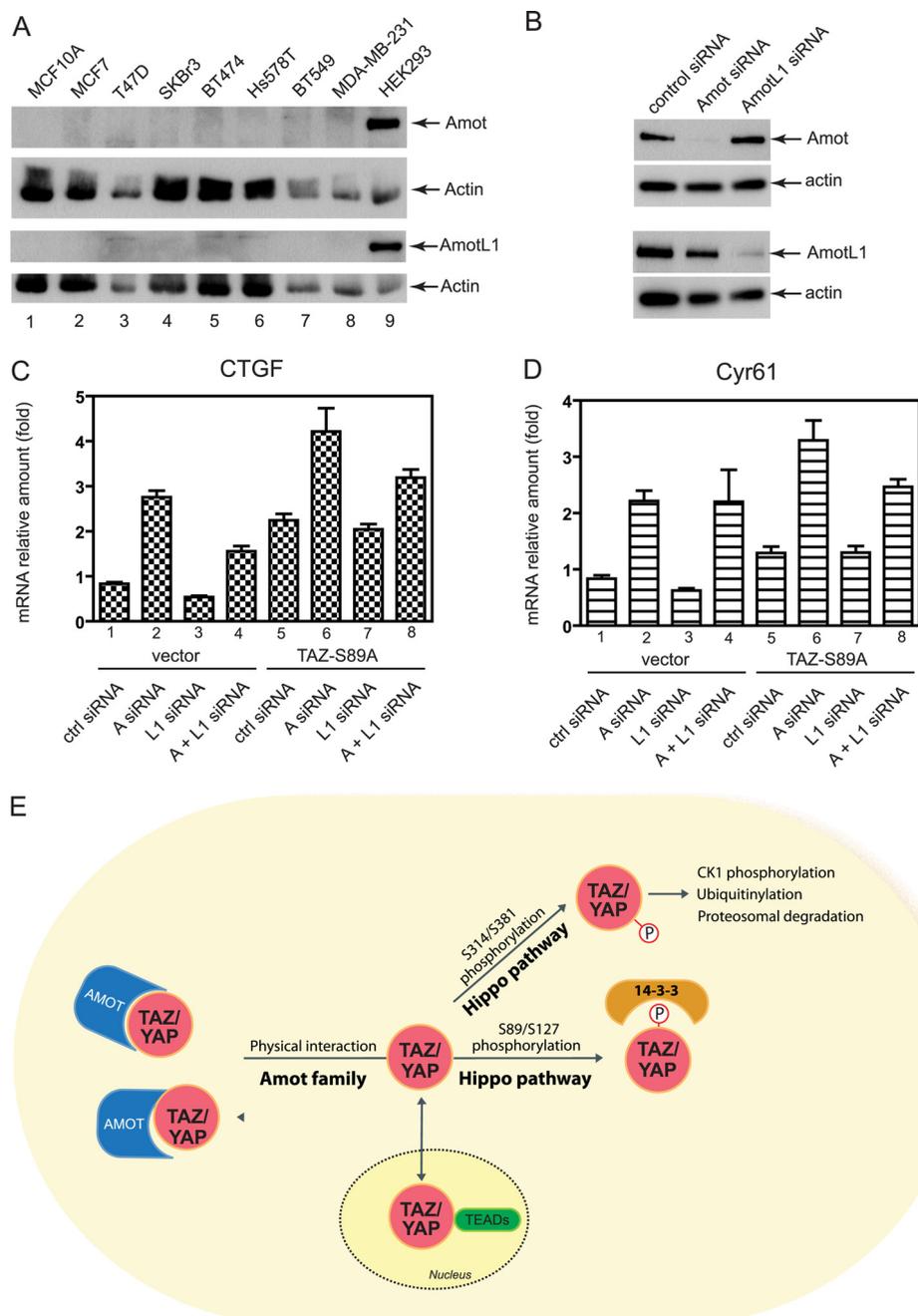
FIGURE 3. **Endogenous Amot but not AmotL1 negatively regulates the expression of endogenous CTGF and Cyr61 genes.** *A*, the lysates derived from the indicated nine different human cell lines were analyzed by immunoblot to detect endogenous Amot (*upper panel*) and AmotL1 (*lower panel*). β-Actin was used as control. *B*, Amot and AmotL1 protein levels in cells transfected with their respective siRNA were determined by immunoblot. *C*, the mRNA levels of CTGF were measured by real-time PCR in HEK293 cells transfected with vector (*columns 1–4*) or TAZ-89A-expressing construct (*columns 5–8*) together with siRNA indicated at the *bottom* (*ctrl siRNA*, control siRNA; *A siRNA*, Amot siRNA; *L1 siRNA*, AmotL1 siRNA; *A+L1 siRNA*, Amot and AmotL1 siRNA together). The levels of CTGF mRNA were normalized to that detected in *column 1*, which was arbitrarily set at 1. *D*, the mRNA levels of Cyr61 were measured in those cells described in *panel C*. The Cyr61 mRNA levels were normalized to that detected in *column 1*, which was arbitrarily set at 1. *Error bars* in *panels C* and *D* indicate S.E. *E*, a working model for diverse regulatory mechanisms for TAZ and YAP. The Hippo pathway causes cytoplasmic sequestration of TAZ and YAP through phosphorylation of Ser[89] and Ser[127], respectively. Furthermore, Hippo pathway-mediated phosphorylation of Ser[314] and Ser[381] leads to further phosphorylation, ubiquitination, and proteasomal degradation of TAZ and YAP, respectively. Interaction with TEADs is important for nuclear accumulation and transcriptional outcome of TAZ and YAP. The results presented in this study suggest that Amot and AmotL1 (likely also AmotL2) function as negative regulators of TAZ and YAP through direct interaction with the WW domain of TAZ and the first WW domain of YAP via the first PP*XY* motif of Amot and AmotL1 that is also conserved in AmotL2, leading to their cytoplasmic retention.

of YAP serve as the binding sites for 14-3-3 proteins. As such, phospho-Ser[89] and phospho-Ser[127] offer the major regulatory effect of the Hippo pathway on the distribution of TAZ and YAP, respectively. Accordingly, S89A and S127A mutants are largely refractory to this inhibition and are mainly distributed

in the nucleus, having much enhanced transcriptional and oncogenic property (10, 12, 20, 23, 31). Recent studies also reveal that the Hippo pathway can regulate the stability of YAP and TAZ (21, 22). Phosphorylation of Ser[381] of YAP and Ser[314] of TAZ by the Hippo pathway primes YAP and TAZ,

respectively, for subsequent phosphorylation by casein kinase 1, leading to their ubiquitination and proteasomal degradation. Therefore, the Hippo pathway can negatively regulate TAZ and YAP by two different mechanisms through cytoplasmic sequestration and proteasomal degradation. Recently, PP2A phosphatase complex was shown to reverse the inhibition imposed on Yorkie by the Hippo pathway (36). Whether there exist other mechanisms regulating TAZ and YAP function is not known.

The results presented in our study offer another novel mechanism of regulation of TAZ and YAP function through direct interaction with Amot and AmotL1, leading to their cytoplasmic sequestration that is independent of Hippo pathway-regulated interaction with 14-3-3 proteins. The general working model derived from our experiments is that Amot and AmotL1 (likely also AmotL2) are primary cytosolic proteins that interact with TAZ and YAP through the first PP*XY* motif of Amot and AmotL1 and the WW domain of TAZ and YAP (Fig. 3*E*). This interaction causes cytoplasmic sequestration of TAZ and YAP in a manner similar to but independent of TAZ-YAP interaction with 14-3-3 proteins. In the case of YAP, it is intriguing to note that first but not the second WW domain is important for interaction with Amot and AmotL1, offering the possibility that the second WW domain of YAP can interact with yet another PP*XY* motif-containing protein, creating more complexity of YAP regulation. The structural basis unique to the first WW domain of YAP that enables its Amot interaction is an interesting issue to be addressed in the future. Because Amot has been shown to be distributed to the junctional complexes of polarized epithelial cells (28), it is also possible that Amot and AmotL1 may mediate localization of TAZ and YAP to the cell junction in polarized epithelial cells. Because the junctional complex is linked to cell-cell adhesion and part of the sensing mode for cell contact, this potential targeting of Amot-TAZ-YAP to the junction may offer an avenue to investigate the potential cross-talk of Amot-mediated regulation and Hippo pathway-mediated control to coordinate cell contact-induced inactivation of TAZ-YAP by the Hippo pathway in epithelial cells. Future experiments should provide insightful understanding about this hypothesis. Another interesting issue that remains to be investigated is whether Amot and AmotL1 (likely also AmotL2) play independent, overlapping, and/or redundant roles in regulating TAZ and YAP. In our overexpression studies in MCF7 cells, overexpression of either Amot or AmotL1 displayed minor inhibition on TAZ target gene expression, whereas expression of both Amot and AmotL1 caused more significant inhibition, suggesting that Amot and AmotL1 may be synergistically and/or additively inhibiting the transcriptional co-activating function of TAZ in MCF7 cells. However, in HEK293 cells, knockdown of Amot alone enhanced expression of TAZ-YAP target genes *CTGF* and *Cyr61* to levels that are comparable with the overexpression of exogenous Hippo refractory S89A mutant of TAZ, suggesting that Amot is the principal member of Amot family in regulating the transcriptional outcome of endogenous TAZ-YAP in terms of *CTGF* and *Cyr61* genes. This possibility is supported by the observation that knockdown of AmotL1 had little impact on *CTGF* and *Cyr61* ex-

pression, whereas simultaneous knockdown of both Amot and AmotL1 had a similar effect as Amot knockdown alone. These results suggest that Amot and AmotL1 may play unique and independent roles in regulating the functional outcome of TAZ-YAP gene expression. As expected, in the presence of S89A, expression of endogenous *CTGF* and *Cyr61* was enhanced. Importantly, under this setting, knockdown of Amot but not AmotL1 further enhanced the expression of *CTGF* and *Cyr61*, suggesting that the exogenous S89A is negatively regulated by endogenous Amot but not AmotL1 with regard to the expression of *CTGF* and *Cyr61*. Therefore, it seems that Amot has a major role in suppressing the activity of endogenous YAP and exogenous TAZ in HEK293 cells. It is also possible that the relative importance of different Amot family members in regulating the function of TAZ-YAP may depend on the cellular context and thus may be different in different cell types because Amot family members may potentially interact with other cellular proteins that indirectly influence their functionality in regulating TAZ-YAP. Another interesting observation is that the expression level of Amot and AmotL1 is relatively low or undetectable in most cell lines examined. Because they are able to negatively regulate the proliferative properties of TAZ-YAP such as *CTGF* and *Cyr61* expression and anchorage-independent growth, low and undetectable expression of Amot and AmotL1 is more favorable for the proliferative requirement of cell lines. This indicates that suppressing the expression of Amot and AmotL1 is one possible mechanism to uncouple TAZ and YAP from their negative regulation. Whether there exists an active mechanism to suppress the expression of Amot and AmotL1 in these cells and how HEK293 cells overcome the potential inhibition of relatively high levels of Amot and AmotL1 expression will be interesting to examine. Along with these lines, the identification of Amot and AmotL1 as novel negative regulators of TAZ-YAP that act independently of the Hippo pathway will provide an exciting opportunity to gain better understanding about not only the role and mechanism of TAZ-YAP but also the integration of TAZ-YAP with diverse regulatory mechanisms.

The mode of restriction of TAZ and YAP by Amot family members is mechanistically similar to the reported inhibition of Yorkie by expanded (Ex) in the fly (37), although Amot and AmotL1 do not display overall sequence homology with Ex. Ex was firstly defined as an upstream regulator to activate Hippo core kinase machinery to restrict Yorkie, but it was recently shown that Ex can also directly interact with Yorkie and thus prevent Yorkie nuclear accumulation, a mode of action that is independent of the Hippo core kinase machinery. Mechanistically, the direct interaction is mediated by the WW domains of Yorkie and PP*XY* motifs of Ex, thus resembling the physical interaction of Amot and AmotL1 with TAZ and YAP.

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