Cell Reports

Inventory of Supplemental Information

Tankyrase inhibitors target YAP by stabilizing angiomotin family proteins

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Supplemental Figures

Figure S1

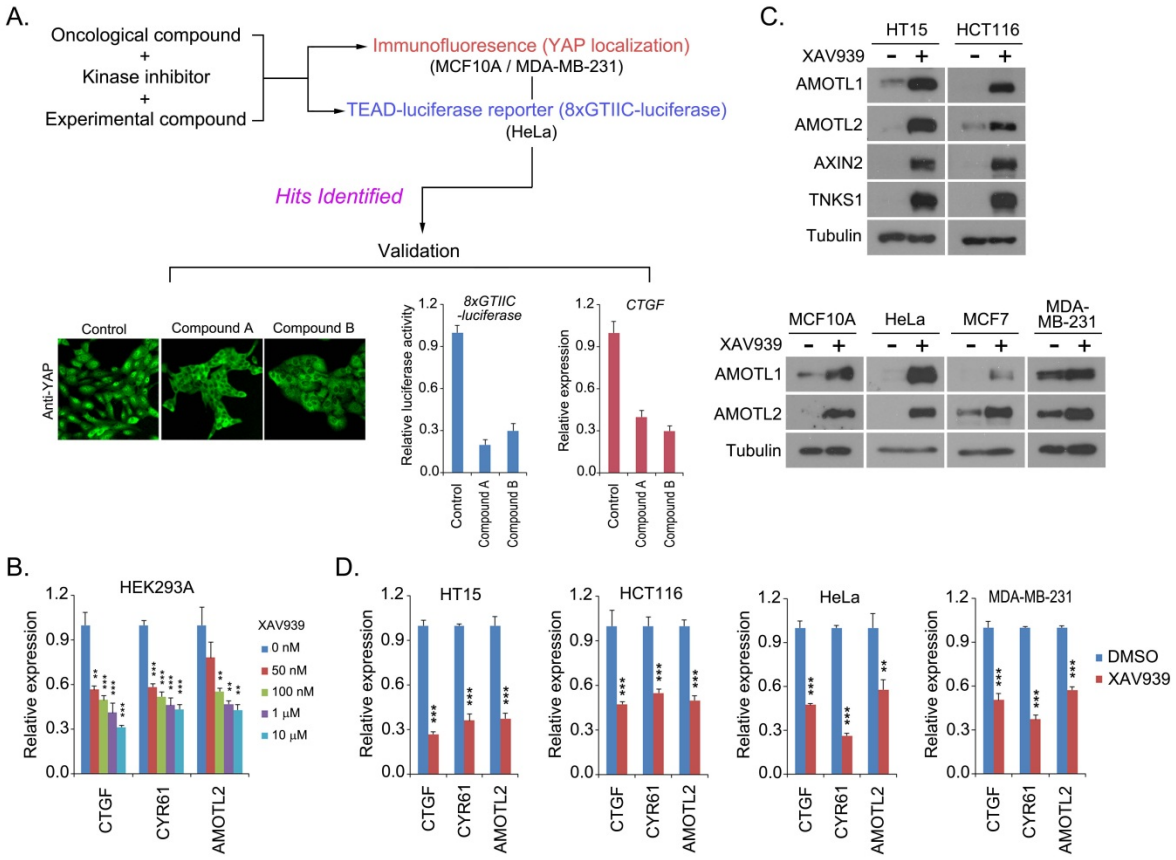

Figure S1. Tankyrase inhibitor XAV939 is identified as a YAP inhibitor (This figure is related to **Figure 1**).

(A) The schematic illustration of the compound screen performed to identify YAP inhibitors. Two compound libraries (oncological compound library and kinase inhibitor library) together with some experimental compounds used in our previous studies were used to perform the screen. YAP immunofluorescent staining and TEAD-luciferase reporter were used to perform the compound screen. Identified hits were further validated individually by YAP immunofluorescence staining, TEAD-luciferase reporter assay and the examination of YAP target gene transcription. (B) XAV939 treatment suppressed YAP transcriptional activity in a

dose-dependent manner. The transcripts of YAP target genes were detected by quantitative PCR in HEK293A cells. Cells were treated with XAV939 at the indicated concentration for 24 hours. (C) XAV939 treatment stabilized AMOT proteins in different cell lines. Western blot was performed with indicated antibodies in dimethyl sulfoxide (control)– and XAV939 (10 μM)–treated cells as indicated for 48 hours. (D) XAV939 treatment suppressed YAP transcriptional activity. Indicated cell lines were treated with 10 μM XAV939 for 48 hours and the transcripts of YAP target genes were detected by quantitative PCR. For all panels, ** $p<0.01$ and *** $p<0.001$.

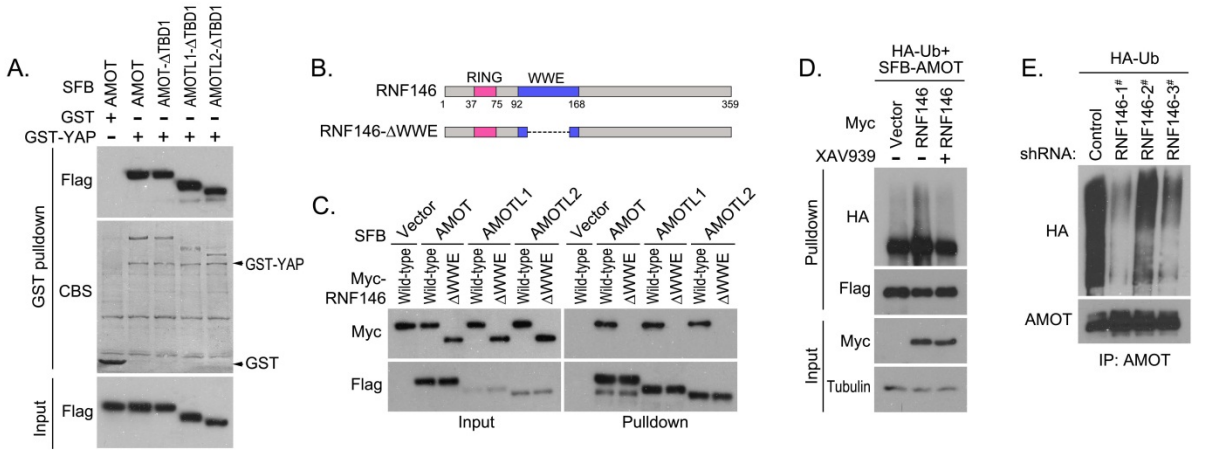

Figure S2. Tankyrase-RNF146 axis promotes the degradation of AMOT proteins (This figure is related to **Figure 3**).

(A) Deletion of tankyrase binding domain (TBD1) in AMOT proteins did not affect the association between AMOT proteins and YAP. Bacterially purified GST-YAP was used for the pulldown experiment. Indicated proteins were detected by Western blot. GST-YAP and GST control were shown by coomassie blue staining (CBS) as indicated by arrows. (B) Schematic illustration of the domain structures for RNF146. (C) RNF146 associated with AMOT proteins through its WWE domain. SFB-tagged AMOT proteins were co-expressed with Myc-tagged RNF146 or its WWE-deleted mutant in HEK293T cells, and cell lysates were subjected to pulldown assays. (D) RNF146 induced the ubiquitination of AMOT protein. HA-tagged ubiquitin (Ub) was co-expressed with indicated proteins in HEK293T cells for 24 h. As indicated, cells were also pretreated by XAV939 (10 μM) for 24hours. Cells were treated with proteasome inhibitor MG132 (10 μM) for 6 h and subjected to pulldown assay. (E) Loss of RNF146 suppressed the ubiquitination of AMOT protein. HA-tagged ubiquitin (Ub) was expressed in HEK293A cells transduced by indicated shRNA for 24 h. Cells were treated with proteasome inhibitor MG132 (10 μM) for 6 h and subjected to immunoprecipitation (IP) assay.

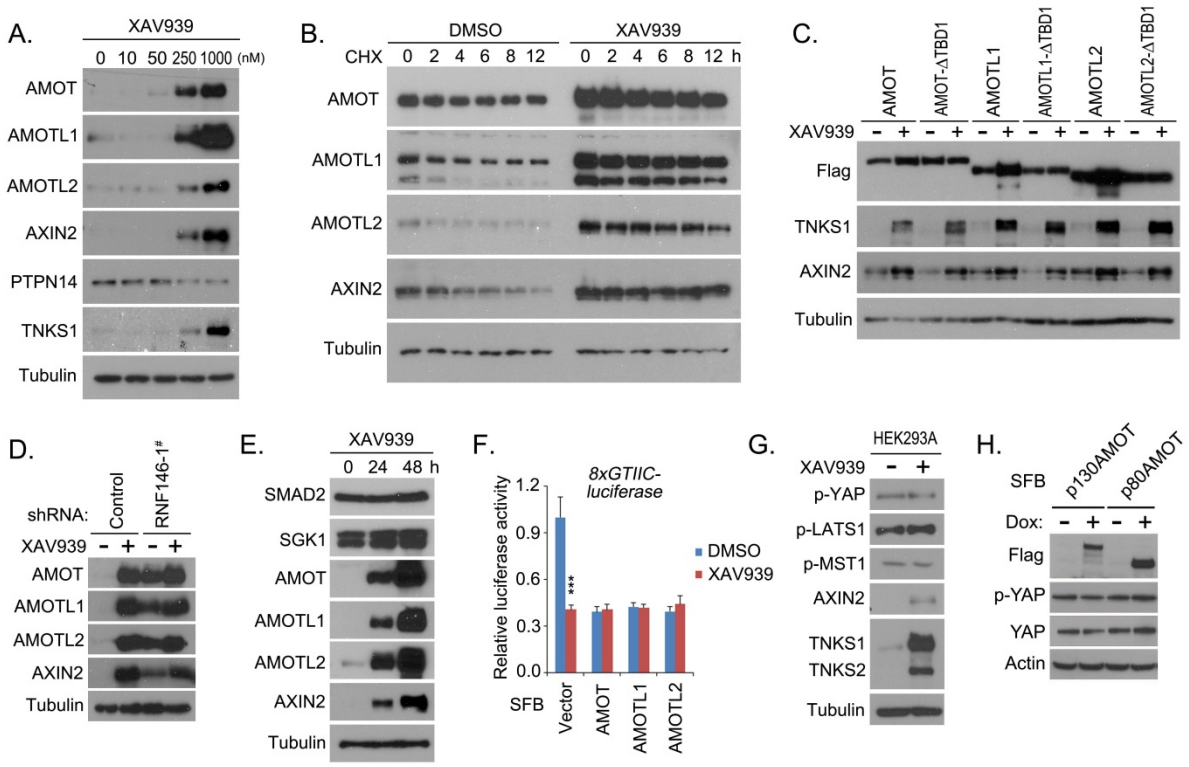

Figure S3. XAV939 targets YAP by stabilizing AMOT proteins (This figure is related to **Figure 4**).

(A) XAV939 treatment increased the levels of AMOT family proteins in a dose-dependent manner. HEK293A cells treated with XAV939 at indicated concentration for 24 hours were collected for Western blot with indicated antibodies. (B) XAV939 treatment stabilized the AMOT proteins. HEK293A cells were pretreated with XAV939 (10 μM) for 24 hours, subjected to cycloheximide (CHX; 100 μg/mL) treatment, and collected at different time points for Western blot with indicated antibodies. (C) XAV939 treatment stabilized wild-type AMOT proteins but not their TBD1-deleted mutants. SFB-tagged AMOT proteins and their TBD1-deleted mutants were expressed in HEK293T cells treated with dimethyl sulfoxide (control) or XAV939 (10 μM) for 24 hours. Western blot was performed with indicated antibodies. (D) Loss

of RNF146 diminished the stabilization effect on AMOT proteins mediated by XAV939 treatment. HEK293A cells transduced with indicated shRNA were treated with dimethyl sulfoxide (control) or XAV939 (10 μM) for 24 hours. Western blot was performed with indicated antibodies. (E) NEDD4.2 E3 ligase did not involve in XAV939-mediated stabilization of AMOT proteins. HEK293A cells were treated with XAV939 (10 μM) for 24 and 48 hours, and western blot was performed with indicated antibodies. (F) Overexpression of AMOT proteins attenuated XAV939-induced suppression of YAP. YAP/TEAD luciferase reporter assay was performed in HeLa cells transfected with vector or indicated SFB-tagged AMOT proteins, and treated with 10 μM XAV939 for 24 h. pSV40-Renilla was used as internal control. (G) XAV939 treatment did not affect YAP phosphorylation or Hippo kinase activation. Western blot was performed in HEK293A cells treated with dimethyl sulfoxide (control) or XAV939 (10 μM) for 24 hours. (H) Overexpression AMOT protein did not affect YAP phosphorylation at Ser127. AMOT proteins were induced to express by doxycycline treatment (Dox, 1μg/mL) for 48 hours and cells were subjected to Western blot with indicated antibodies. *** $p<0.001$

Figure S4

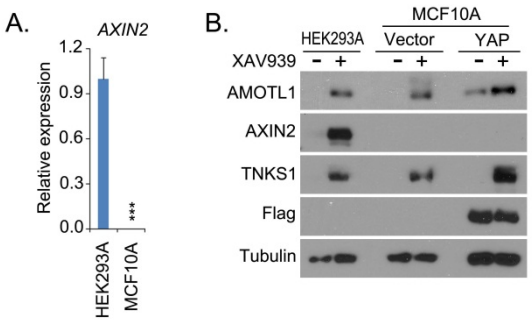

Figure S4. XAV939 targeted YAP-transformed MCF10A cells independent of the Wnt pathway (This figure is related to **Figure 5**).

(A) Wnt activity was relatively low in MCF10A cells. The transcript of *AXIN2* was detected by quantitative PCR in indicated cells. (B) AXIN2 protein was hard to be detected in vector control MCF10A cell and YAP overexpressing MCF10A cell. Indicated cell lines were treated with dimethyl sulfoxide (control) or XAV939 (10 μM) for 24 hours. Western blot was performed with indicated antibodies. *** $p<0.001$

Table S1. Identification of tankyrase 1-associated protein complex (This table is related to **Figure 2**).

Tankyrase 1(TNKS1)-associated protein complex was isolated from HEK293T cells stably expressing SFB tagged TNKS1 and identified through mass spectrometry analysis. Identified protein and peptide information were listed.

Supplemental Experimental Procedures

Antibodies and chemicals

Anti-YAP antibody was raised by immunizing rabbits with bacterially expressed and purified GST-fused human full-length YAP protein. Anti-AMOTL2 antibody was raised by immunizing rabbits with bacterially expressed and purified GST-fused human AMOTL2 protein (amino acids 1~675). Anti-PTPN14 antibody was raised by immunizing rabbit with bacterially expressed and purified GST-PTPN14 fusion protein containing the 400~800 amino acids of human PTPN14. All the antisera were affinity-purified by using the AminoLink Plus immobilization and purification kit (Pierce). Additional anti-YAP (sc101199) antibody was purchased from Santa Cruz Biotechnology. Anti-AMOTL1 (HPA001196), anti-α-tubulin (T6199-200UL), and anti-flag (M2) (F3165-5MG) monoclonal antibodies were obtained from Sigma-Aldrich. Anti-myc (sc-40) and anti-tankyrase1/2 (H350) antibodies were purchased from Santa Cruz Biotechnology. Anti-HA (16B12) monoclonal antibody was obtained from Covance. Anti-LATS1 (9153S), anti-LATS2 (13646S), anti-MST1 (3682S), anti-MST2 (3952S), anti-AXIN2 (2151S), anti-KIBRA (8774S), anti-SAV1 (3507S), anti-Biotin (5571S), and anti-NF2 (6995S), anti-SMAD2 (5339S), anti-SGK1 (12103S), anti-phospho-YAP (S127) (4911S), anti-phospho-LATS1 (Thr1079) (8654S), and anti-phospho-MST1 (Thr183)/MST2 (Ser180) (3681S) polyclonal antibodies polyclonal antibodies were purchased from Cell Signaling Technology. Anti-TEAD1 (610922) monoclonal antibody was obtained from Millipore. Anti-PAR polymer monoclonal antibody (4335-MC-100) was purchased from Trevigen.

Tankyrase inhibitors XAV939 and WIKI4, proteasome inhibitor MG132, doxycycline and cycloheximide were purchased from Sigma-Aldrich. PARP1/2 inhibitor olaparib was

obtained from LC Laboratories. Tankyrase inhibitor JW55 and PARG inhibitor ADP-HPD were obtained from Millipore. Growth factor– reduced basement membrane matrigel matrix (#354230) was purchased from Corning.

Cell culture and transfection

HEK293T and HeLa cells were purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2 (v/v). HEK293A, HT15 and HCT116 cells were kindly provided by Dr. Jae-Il Park (MD Anderson Cancer Center). MCF7 and MDA-MB-231 were purchased from American Type Culture Collection (ATCC) and kindly provided by Dr. Mien-Chie Hung (MD Anderson Cancer Center). MCF10A cells were purchased from ATCC and kindly provided by Dr. Dihua Yu (MD Anderson Cancer Center). MCF10A cells were maintained in DMEM/F12 medium supplemented with 5 % horse serum, epidermal growth factor (200 ng/mL), hydrocortisone (500 ng/mL), cholera toxin (100 ng/mL), and insulin (10 μg/mL) at 37°C in 5% CO2 (v/v). All culture media contained 1% penicillin and streptomycin antibiotics. Plasmid transfection was performed with the polyethylenimine reagent.

Constructs and viruses

All constructs were generated by polymerase chain reaction (PCR) and subcloned into pDONOR201 vector using Gateway Technology (Invitrogen) as the entry clones. As needed, the entry clones were subsequently recombined into gateway-compatible destination vectors for the expression of *N*- or *C*-terminal tagged fusion proteins. The 8xGTIIC-luciferase reporter (Plasmid

#34615) was purchased from Addgene. PCR-mediated site-directed mutagenesis was used to generate serial deletions as indicated.

HA-Flag–tagged retrovirus for YAP was generated by co-transfecting with pCL-Ampho packaging vector in the BOSC23 cell line. Cells were infected twice by 0.45 μm–filtered retrovirus with the addition of polybrene (8 μg/mL), and the stably transfected cells were selected with puromycin (2 μg/mL). Pooled stable cells were used for all the experiments after validation by immunostaining and Western blotting.

The SFB-lentiviral expression vector was generated by inserting the gateway response fragment (attR1-ccdB-attR2)–fused SFB tag into the *XbaI* and *SwaI* multi-clonal sites of the pCDH-CMV-EF1-GFP vector (kindly provided by Dr. M. James You, MD Anderson Cancer Center). TNKS1 was cloned into this vector through a gateway-based LR reaction.

TNKS1 and AMOTL1 pLKO-shRNAs were purchased from Sigma-Aldrich: AMOTL1 (TRCN0000127557) and TNKS1 (TRCN0000040187). TNKS2, AMOTL2 and RNF146 pGIPZ-shRNAs were obtained from the shRNA-ORFeome Core Facility at MD Anderson Cancer Center: TNKS2 (V3LHS_354389), AMOTL2 (V2LHS_229971), RNF146-shRNA-1# (V3LHS_395653), RNF146-shRNA-2# (V3LHS_395650), and RNF146-shRNA-3# (V2LHS_12922). Control shRNA sequence is 5′-TCTCGCTTGGGCGAGAGTAAG-3′.

All lentiviral supernatants were generated by transient transfection of HEK293T cells with helper plasmids pSPAX2 and pMD2G (kindly provided by Dr. Zhou Songyang, Baylor College of Medicine) and harvested 48 hours after transfection. Supernatants were passed through 0.45μm filters and used to infect cells with the addition of polybrene (8 μg/mL).

Compound library screen

Two compound libraries (oncological compound library including 144 compounds and kinase inhibitor library including 591 compounds) in 96-well plate format with 11 μM concentration for each compound were generously provided by Dr. Steven H. Lin (MD Anderson Cancer Center). Besides, we also include some experimental compounds used in our previous studies to perform this screen. As for YAP-immunofluorescence screen, sparse or confluent MCF10A cells were chosen and seeded in 96-well optical bottom plate (12-566-70, Fisher Scientific). Cells were treated with compound library at 0.5 μM for 24 hours. Immunostaining were performed within the 96-well plate and pictures were automatically captured by ImageXpress^{Micro} High-Content Imaging Systems (Molecular Devices). Four different fields were chosen to take pictures for each well with both YAP and Dapi staining. MDA-MB-231 cells were used to repeat the screen similar to that of sparse MCF10A cells. In the meantime, TEAD-luciferase reporter assay was used to perform the screen with the same library. TEAD-luciferase reporter 8xGTIIC was transiently expressed in HeLa cells with pSV40-Renilla for 24 hours and treated by compound library at 0.5 μM for 24 hours. Cells were lysed and subjected to luciferase reporter assay by using Dual-Luciferase® Reporter Assay System (Promega). The hits identified in both screens were taken to perform the further validations.

Tandem affinity purification of SFB-tagged protein complexes

HEK293T cells were infected twice with SFB-TNKS1 lentivirus. Pooled cells stably expressing the tagged TNKS1 were selected by culturing in medium containing puromycin (2 μg/mL), and the protein expression was confirmed by immunostaining and Western blotting.

Affinity purification was performed as described previously (Wang et al., 2014). Briefly, TNKS1-SFB HEK293T stable cells were subjected to lysis in NETN buffer (100 mM NaCl, 20

mM Tris-HCl, 0.5 mM EDTA. 0.5 % Nonidet P-40) with protease and phosphatase inhibitors at 4°C for 20 minutes. Crude lysates were subjected to centrifugation at 4°C and 14,000 rpm for 15 minutes. Supernatants were incubated with streptavidin-conjugated beads (Amersham) for 4 hours at 4°C. The beads were washed three times with NETN buffer, and bound proteins were eluted with NETN buffer containing biotin (2 mg/mL; Sigma) overnight at 4°C. The elutes were incubated with S protein beads (Novagen) for 4 hours. The beads were washed three times with NETN buffer and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Protein bands were excised and subjected to mass spectrometry analysis (performed by the Taplin Mass Spectrometry Facility, Harvard Medical School).

Mass spectrometry analysis

Excised gel bands were cut into approximately 1 mm^3 pieces. Gel pieces were then subjected to in-gel trypsin digestion (Shevchenko et al., 1996) and dried. Samples were reconstituted in 5 µl of HPLC solvent A (2.5% acetonitrile, 0.1% formic acid). A nano-scale reverse-phase HPLC capillary column was created by packing 5 µm C18 spherical silica beads into a fused silica capillary (100 µm inner diameter x ~20 cm length) with a flame-drawn tip. After equilibrating the column each sample was loaded via a Famos autosampler (LC Packings, San Francisco CA) onto the column. A gradient was formed and peptides were eluted with increasing concentrations of solvent B (97.5% acetonitrile, 0.1% formic acid).

As peptides eluted, they were subjected to electrospray ionization and then entered into an LTQ Velos ion-trap mass spectrometer (ThermoFisher, San Jose, CA). Peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Peptide sequences (and hence protein identity) were determined by matching protein

databases with the acquired fragmentation pattern by the software program, SEQUEST (ver. 28) (ThermoFisher, San Jose, CA). Enzyme specificity was set to partially tryptic with 2 missed cleavages. Modifications included carboxyamidomethyl (cysteines, fixed) and oxidation (methionine, variable). Mass tolerance was set to 2.0 for precursor ions and 1.0 for fragment ions. The database searched was the Human IPI databases version 3.6. Because we used HEK293T cells, the Human IPI database was used. The number of entries in the database was 160,900 which included both the target (forward) and the decoy (reversed) human sequences. Spectral matches were filtered to contain less than 1% FDR at the peptide level based on the target-decoy method (Elias and Gygi, 2007). Finally, only tryptic matches were reported and spectral matches were manually examined. When peptides matched to multiple proteins, the peptide was assigned so that only the most logical protein was included (Occam's razor). This same principle was used for isoforms when present in the database. The longest isoform was reported as the match.

Immunofluorescent staining

Immunofluorescent staining was performed as described previously (Wang et al., 2008). Briefly, cells cultured on coverslips were fixed by 4% paraformaldehyde for 10 minutes at room temperature and then extracted with 0.5% Triton X-100 solution for 5 minutes. After blocking with Tris-buffered saline and tween 20 solution containing 1% bovine serum albumin, cells were incubated with indicated primary antibodies for 1 hour at room temperature. After that, cells were washed and incubated with fluorescein isothiocyanate or rhodamine-conjugated second primary antibodies (Jackson ImmunoResearch) for 1 hour. Cells were counterstained with 100 ng/mL 4′,6-diamidino-2-phenylindole (DAPI) for 2 minutes to visualize nuclear DNA. The cover slips were mounted onto glass slides with anti-fade solution and visualized under a Nikon

ECLIPSE E800 fluorescence microscope with a Nikon Plan Fluor 60× oil objective lens (NA 1.30).

RNA extraction, reverse transcription and real-time PCR

RNA samples were extracted with TRIZOL reagent (Invitrogen). Reverse transcription assay was performed by using the ProtoScript M-MuLV Taq RT-PCR Kit (New England Biolabs) according to the manufacturer's instructions. Real-time PCR was performed by using Power SYBR Green PCR master mix (Applied Biosystems). For quantification of gene expression, the $2^{-\Delta\Delta Ct}$ method was used. GAPDH expression was used for normalization. The sequence information for each primer used for gene expression analysis was as follows:

GAPDH-Forward: 5′-ATGGGGAAGGTGAAGGTCG-3′

GAPDH-Reverse: 5′-GGGGTCATTGATGGCAACAATA-3′

CTGF-Forward: 5′-CCAATGACAACGCCTCCTG-3′

CTGF-Reverse: 5′-GAGCTTTCTGGCTGCACCA-3′

CYR61-Forward: 5′-AGCCTCGCATCCTATACAACC-3′

CYR61-Reverse: 5′-GAGTGCCGCCTTGTGAAAGAA-3′

AMOT-Forward: 5′- CCTTCAGGGAGCTGCTAAGA -3′

AMOT-Reverse: 5′- GAGTTCCTGGCTGACAATGG -3′

AMOTL1-Forward: 5′- GAACTAGCCATGATCGCCTC -3′

AMOTL1-Reverse: 5′- ACCTGGACAGGACTACTGGG -3′

AMOTL2-Forward: 5′-AGCTTCAATGAGGGTCTGCT-3′

AMOTL2-Reverse: 5′-TGAAGGACCTTGATCACTGC-3′

TNKS1-Forward: 5′- GACCCAAACATTCGGAACAC-3′

TNKS1-Reverse: 5′- GCAGCTTCTAGGAGTTCGTCTT-3′

TNKS2-Forward: 5′- ACGTGGAACGAGTCAAGAGG-3′

TNKS2-Reverse: 5′- TTGCACCATTCTGAAGCAAA-3′

RNF146-Forward: 5′- GAGAAAAGACTGCGAGGTGG -3′

RNF146-Reverse: 5′- GATGCCTGCCACAAAAATAAA-3′

GST pulldown assay

GST-fused YAP was expressed and purified in *Escherichia coli* BL21 cells. GST-YAP protein (2 μg) was immobilized on GST-sepharose 4B beads and incubated with various cell lysates for 2 hours at 4°C. Beads were washed three times. Proteins bound to beads were eluted and subjected to SDS-PAGE and Western blotting analysis.

Supplemental References

Elias, J.E., and Gygi, S.P. (2007). Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nat Methods *4*, 207-214.

Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem *68*, 850-858.

Wang, W., Chen, L., Ding, Y., Jin, J., and Liao, K. (2008). Centrosome separation driven by actin-microfilaments during mitosis is mediated by centrosome-associated tyrosine-phosphorylated cortactin. J Cell Sci *121*, 1334-1343.

Wang, W., Li, X., Huang, J., Feng, L., Dolinta, K.G., and Chen, J. (2014). Defining the protein-protein interaction network of the human hippo pathway. Mol Cell Proteomics *13*, 119-131.