Supplemental Data

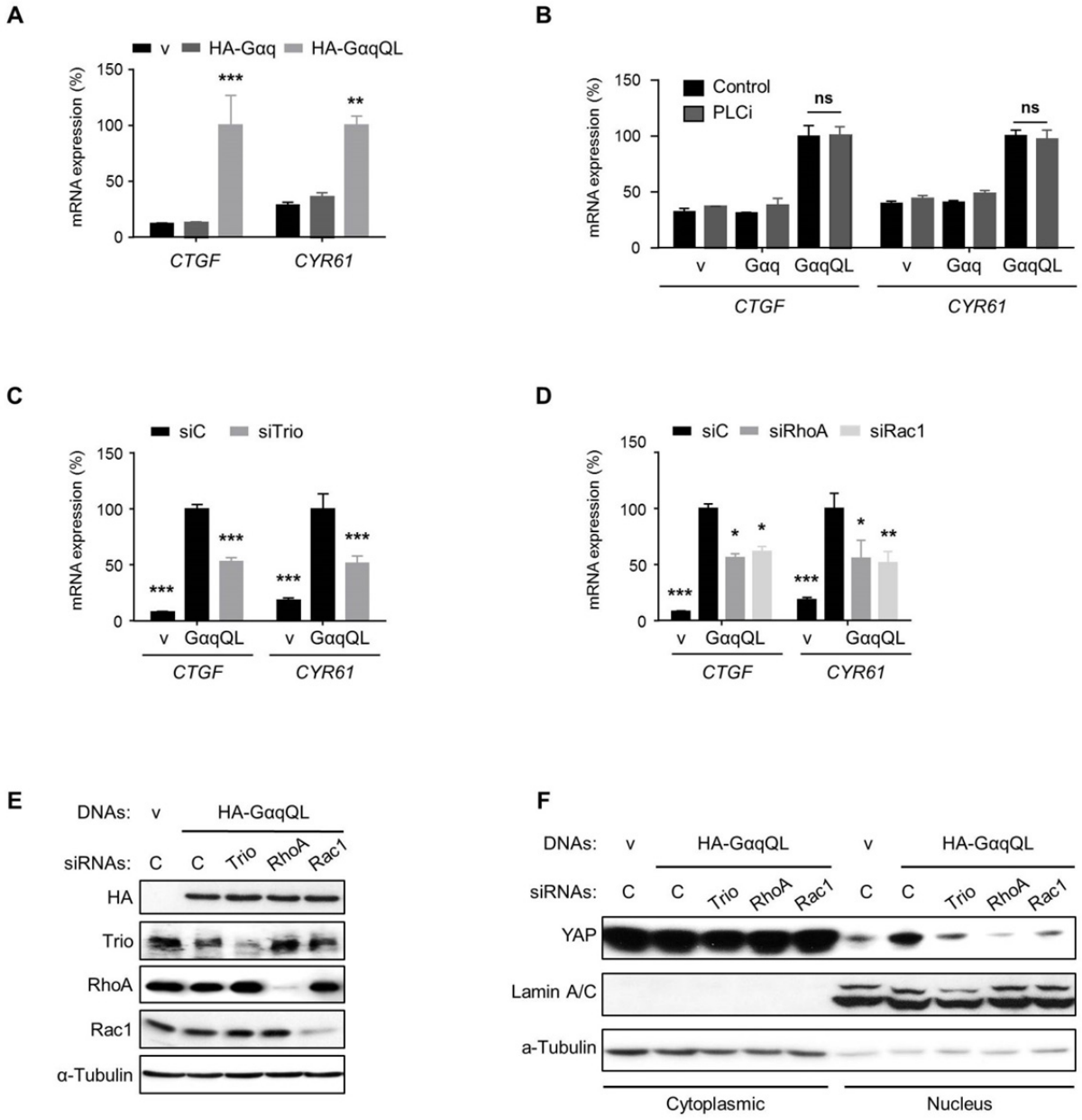

Figure S1 Related to Figure1

(A) HEK293 cells were transfected vector, HA-Gαq or HA-GαqQL, followed by Q-PCR assay to test YAP-regulated genes (*CTGF* and *CYR61*) (mean ± SEM, n = 3). (B) HEK293 cells were transfected with HA-Gαq or HA-GαqQL, followed by PLCi treatment (6 hr) and Q-PCR assay to test YAP-regulated genes (*CTGF* and *CYR61*) (mean ± SEM, n = 3). (C) HEK293 cells were

cotransfected with siRNA Trio or control and HA-GαqQL or vector, followed by Q-PCR assay to test YAP-regulated genes (*CTGF* and *CYR61*) (mean ± SEM, n = 3). (D) HEK293 cells were cotransfected with siRNAs RhoA, Rac1 or control and HA-GαqQL or vector, followed by Q-PCR assay to test YAP-regulated genes (*CTGF* and *CYR61*) (mean ± SEM, n = 3). (E) Western blot analysis of the indicated proteins show HA-GαqQL expression and that the specific siRNAs reduced the expressions of their targeted genes in HEK293. HEK293 cells were cotransfected with siRNAs Trio, RhoA, Rac1 or control and HA-GαqQL or vector control. (F) Western blot shows that the accumulation of YAP in the nuclear fraction caused by GαqQL requires Trio, RhoA and Rac1, using the enrichment in Lamin A/C and α-Tubulin as nuclear and cytoplasmic markers, respectively.

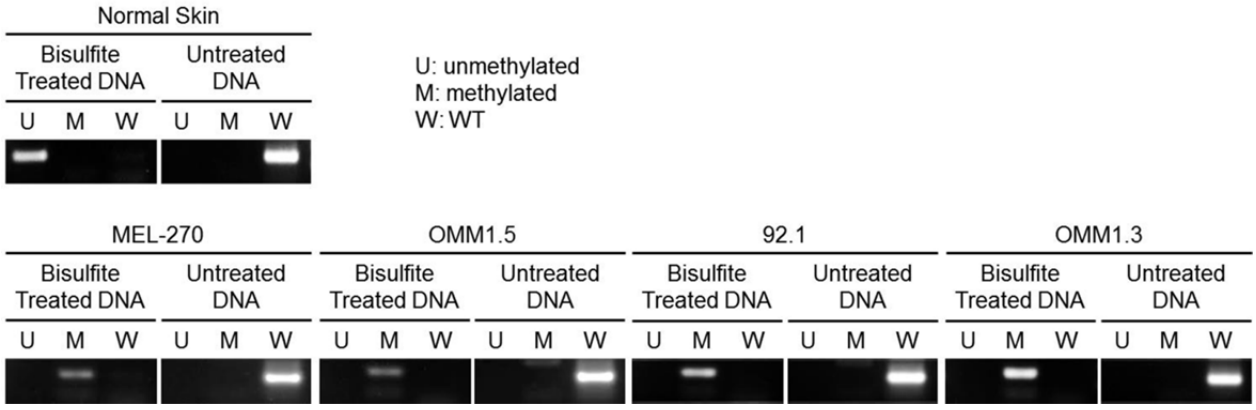

Figure S2 Related to Figure 2. DNA methylation of *CDKN2A* (*p16^{ink4}*) promoter in representative uveal melanoma cell lines (MEL-270, OMM1.5, 92.1 and OMM1.3), using normal skin as control.

Methylation-specific PCR (MSP) analysis was performed with primer sets specific for the unmethylated (U), methylated (M), or unmodified/wild-type (W) *CDKN2A* promoter region.

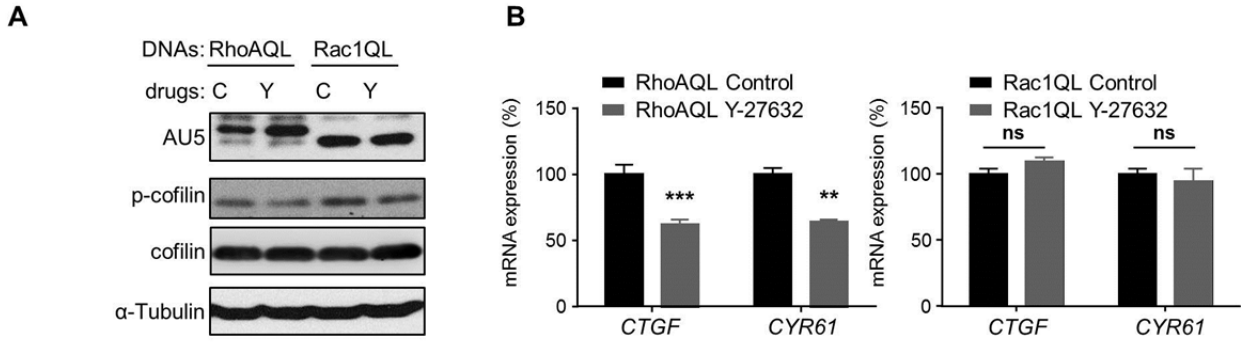

Figure S3 Related to Figure 4

(A) HEK293 cells were transfected with RhoAQL or Rac1QL, followed with Y-27632 treatment and Western blot analysis for AU5, p-cofilin, cofilin and α-Tubulin as a loading control. (B) HEK293 cells were transfected with RhoAQL or Rac1QL, followed with Y-27632 treatment and Q-PCR assay to test YAP-regulated genes (*CTGF* and *CYR61*) (mean ± SEM, n = 3).

Supplemental Experimental Procedures

Cell lines, culture procedures, and chemicals: HEK293 cells were cultured in DMEM (Invitrogen, CA) containing 10% FBS (Sigma-Aldrich Inc., MO) and 1× antibiotic/antimycotic solution (Sigma-Aldrich Inc., MO). Uveal melanoma OMM1.3, OMM1.5, Mel270 and 92.1 cells and cutaneous melanoma WM-266 and SK-mel-2 cells have been described elsewhere (Schmitt et al., 2007; Zuidervaart et al., 2005). To establish cells expressing shRNA control or human shRNA-Trio in HEK293 cells, and shRNA control or human shRNA-YAP in OMM1.3 cells, the cells were infected with the corresponding lentiviral (shRNAs) supernatants for 16 hours. After that, the cells were returned to normal growth medium. Infected cells were selected with puromycin (1μg/ml for HEK293, 0.5μg/ml for OMM1.3). Lentiviral stocks were prepared and titrated with HEK293T cells as the packaging cells as previously reported (Basile et al., 2004). Y-27632 (Tocris Cookson Inc., MO) (10μM) and Latrunculin A (Lat.A) (1μM) were used to treat uveal melanoma cells for 1 and 6 h, followed by immunofluorescence and qPCR, respectively. Verteporfin (VP) (CAS number: 129497-78-5; USP Reference Standards, Rockville, MD) was dissolved in DMSO to make a 100mg/ml stock solution.

siRNA: All human siRNA sequences and providers are described in the following table. All human cells were transfected when 40% confluent using Lipofectamine RNAiMAX Reagent (Invitrogen, CA) according to manufacturer's instructions, using 50nM of each siRNA, and experiments were performed, when the cells were confluent.

Details of RNAi and oligonucleotide sequences used in this study.

shRNA construct	**Source**	**Homology**	**Oligo ID**
pGIPZ-sh-Control	Open Biosystems	-	-

pGIPZ-shTrio-A	Open Biosystems	Homo sapiens	V2LHS_1430
pGIPZ-shTrio-B	Open Biosystems	Homo sapiens	V2LHS_1432
pTRIPZ-sh-Control	Open Biosystems	-	-
pTRIPZ-H-shYAP#2	Open Biosystems	Homo sapiens	V2THS_247011
pTRIPZ-H-shYAP#1	Open Biosystems	Homo sapiens	V2THS_65509

siRNA	**Source**	**Homology**	**Details**
Non-Targeting	Thermo Scientific	-	-
Hs_GNAQ	Thermo Scientific (Smart pool)	Homo sapiens	Human (GNAQ 2776)
Hs_Trio	Thermo Scientific (Smart pool)	Homo sapiens	Human (TRIO 7204)
Hs_RhoA	Thermo Scientific (Smart pool)	Homo sapiens	Human (RHOA 387)
Hs_Rac1	Thermo Scientific (Smart pool)	Homo sapiens	Human (RAC1 5879)
siRNA Control	Sigma Aldrich	-	SIC001
Hs_LATS1	Sigma Aldrich	Homo sapiens	Hs01_00046128
Hs_LATS2	Sigma Aldrich	Homo sapiens	Hs01_00158803
Hs_AMOT	Thermo Scientific (Smart pool)	Homo sapiens	Human (AMOT 154796)

Oligonucleotides	**Homology**	**Sequence**
Hs. GAPDH fw	Homo sapiens	5'-GAGTCAACGGATTTGGTCGT-3'
Hs. GAPDH rev	Homo sapiens	3'- TTGATTTTGGAGGGATCTCG-5'
Hs. CTGF fw	Homo sapiens	5'-GTTTGGCCCAGACCCAACTA-3'
Hs. CTGF rev	Homo sapiens	5'-GGCTCTGCTTCTCTAGCCTG-3'
Hs. CYR61 fw	Homo sapiens	5'-CAGGACTGTGAAGATGCGGT-3'
Hs. CYR61 rev	Homo sapiens	5'-GCCTGTAGAAGGGAAACGCT-3'
Hs. p16-W fw	Homo sapiens	5'-CAGAGGGTGGGGCGGACCGC-3'
Hs. p16-W rev	Homo sapiens	5'-CGGGCCGCGGCCGTGG-3'
Hs. p16-M fw	Homo sapiens	5'-TTATTAGAGGGTGGGGCGGATCGC-3'
Hs. p16-M rev	Homo sapiens	5'-GACCCCGAACCGCGACCGTAA-3'
Hs. p16-U fw	Homo sapiens	5'-TTATTAGAGGGTGGGGTGGATTGT-3'
Hs. p16-U rev	Homo sapiens	5'-CAACCCCAAACCACAACCATAA-3'

DNA constructs: Plasmids pCEFL-HA, pCEFL-HA-Gαq, pCEFL-HA-GαqQL pCEFL-AU5, pCEFL-AU5-RhoAQL and pCEFL-AU5-Rac1QL were described previously (Marinissen et al., 2003; Teramoto et al., 2003) . shRNA constructs were purchased from Open Biosystems; pGIPZ-sh-Control-IRES-GFP-Puro was used as control for Trio knockdown. Vectors containing shRNA sequences to knockdown human Trio were described in the previous table. pTRIPZ-sh-Control-IRES-Tomato-Puro was used as control for YAP knockdown. Vectors containing

shRNA sequences to knockdown human YAP were described in the previous table. pCMX-Gal4-TEAD4 and pGL4.23-5×GAL4-binding UAS promoter luciferase have been described (Zhao et al., 2008). Luciferase normalization was performed by co-transfecting pRL-Null vector (Promega, Madison, WI).

Statistical analysis: All data analysis was performed using GraphPad Prism version 6 for Windows (GraphPad Software, CA). The data were analyzed by ANOVA test or t-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Human tumor xenografts and VP *in vivo* treatment: Female NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1wjl}/SzJ mice (commonly known as NOD *scid* gamma, Jackson Laboratory, Maine), 5 to 6 weeks of age and weighing 18 to 20g, were used in the study of OMM1.3 cells, housed in appropriate sterile filter-capped cages, and provided food and water ad libitum. All procedures were essentially as previously described (Amornphimoltham et al., 2004; Vaque et al., 2013). Briefly, exponentially growing cultures were harvested, washed, resuspended in RPMI 1640, and 1.5 x 10^6 viable cells were transplanted subcutaneously into the flanks of mice. For tumor growth analysis, tumor volume was assessed as [($LW2/2$); where L and W represent the length and the width of the tumor]. The animals were monitored twice weekly for tumor development. Results of animal experiments were expressed as mean ± SEM of a total of 6 tumors analyzed. To administer VP to mice, the chemical was first dissolved in DMSO to make a 100mg/ml stock. PBS was then used as the solvent to make 10mg/ml injection suspension by vigorous rocking by vortex. Due to its limited aqueous solubility, the resulting VP preparation is a chemical suspension. The VP

suspension was administered intraperitoneally at a dose of 100mg/kg, using 10% DMSO in PBS as control (Liu-Chittenden et al., 2012).

Mice: FVB/N mice carrying the cytokeratin 5 promoter in the reverse tetracycline transactivator (rtTA) (*k5-tet-on*) and have been previously described (Gunther et al., 2003; Vitale-Cross et al., 2004). For the generation of *tet-HA-GαqQL* transgenic mice, HA-GαqQL coding sequence was cloned downstream of the seven tet-responsive element (tetO7) in a modified pBSRV vector (Gogos et al., 2000). The fragment containing the *TetO–HA-GαqQL* expression cassette was isolated by PmeI digestion from vector sequences and purified for microinjection into fertilized oocytes. Founders were identified for the presence of the transgene by screening genomic DNA from tail biopsies using a PCR reaction. All transgenic lines were generated and maintained in a FVB/N background. The concentrations of doxycycline in the food grain-based pellets (Bio-Serv, Frenchtown, NJ) was 6g/kg. *Dct-rtTA* transgenic mice were used to target the expression of *tet*-regulated genes to melanocytes, and the *TRE (tet)-H2BGFP* transgenic mice to identify the *rtTA* positive cells (Zaidi et al., 2011). We also used $p16^{Ink4a}$ and $p19^{Ink4b}$ (*p16p19*KO) mice, which is defective in the *Ink4* tumor suppressor gene locus. All transgenic lines are on an FVB/N background. The *p16p19*KO mice obtained from the NCI Mouse Repository (Strain Code: 01XB1) in a C57BL/6J background and crossed multiple times with the transgenic FVB/N mice. All experiments were conducted using littermate controls.

Small GTPases activation assays: RhoA and Rac1 activity was assessed by a modified method described previously (Patel et al., 2007). Briefly, cells were treated as indicated and lysed at 4°C in a buffer containing 20mM Hepes, pH 7.4, 0.1M NaCl, 1% Triton X-100, 10mM EGTA,

40mM β-glycerophosphate, 20mM $MgCl_2$, 1 mM Na_3VO_4 and 1x anti-protease cocktail (Sigma-Aldrich Inc., MO). Lysates were incubated with Rhotekin-RBD protein agarose beads (Cytoskeleton INC., CO) or glutathione S-transferase beads (Amersham, NJ) coupled to the GST-CRIB domain PAK1, or GST-Ras Binding Domain. Associated GTP-bound proteins were released with protein loading buffer, boiled and subjected to 15% acrylamide SDS-PAGE followed by western blot analysis using anti-RhoA (C-18, Santa Cruz Biotech., CA) and anti-Rac1 (BD Biosciences, CA).

Immunoblot assays: Western blot assays were performed as described previously (Vaque et al., 2013). Primary antibodies were diluted 1/1000, unless otherwise stated. (anti-Gαq (E-17; Santa Cruz Biotech., CA), anti-RhoA (C-18; Santa Cruz Biotech., CA), anti-Rac1 (BD Biosciences, CA), anti-GAPDH (14C10; Cell Signaling Technology, MA), anti-Trio (H120; Santa Cruz Biotech., CA), anti-YAP (Cell Signaling Technology, MA), anti-pYAP (S127) (Cell Signaling Technology, MA), anti-LATS1 (Cell Signaling Technology, MA), anti-LATS2 (Bethyl), anti-α-Tubulin (Cell Signaling Technology, MA), anti-Lamin A/C (Santa Cruz Biotech., CA), anti-p-cofilin (Cell Signaling Technology, MA), anti-cofilin (Cell Signaling Technology, MA), anti-AMOT (Abcam, MA), anti-HA tag antibody (Covance, CA), anti-AU5 tag antibody (Covance, CA) and anti-flag tag antibody (Covance, CA). Secondary antibodies were diluted 1/40,000; Goat anti-rabbit and anti-mouse secondary antibodies coupled to horseradish peroxidase were purchased from Southern Biotech (Birmingham, AL). Western blots were developed using Immobilon Western reagent (Millipore, MA) according to the manufacturer's instructions. Antibodies from the Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa were maintained with funding from the NICHD. The phos-tag reagents were purchased from

Wako Chemicals, and gels containing phos-tag were prepared according to the manufacture's instructions.

Clinical samples: Snap frozen uveal melanoma tissues were generously provided by Dr. James T. Handa and Dr. Shannath Merbs, Wilmer Eye Institute, Johns Hopkins School of Medicine; tissue was obtained from consenting patients in accordance with an Institutional Review Board approved study. Normal skin samples were purchased from US Biomax and Biochain.

Immunofluorescence: Cells cultured on poly-L-lysine-coated coverslips and frozen tissues sections were washed with PBS to remove the OCT, were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 30 min, and permeabilized using 0.05% Triton X-100 for 10 min. The cells were blocked with 3% FBS-containing PBS for 30 min, and incubated with the indicated antibodies (in 3% FBS-PBS otherwise stated) for 1h at room temperature. The reaction was visualized with Alexa-labeled secondary antibodies (Invitrogen, CA). Samples were mounted in PBS buffer containing Hoechst 33342 (Molecular Probes, OR) for nuclear staining. Images were acquired with an Axio Imager Z1 microscope equipped with ApoTome system controlled by ZEN 2012 software (Carl Zeiss, NY). Antibodies used were: anti-YAP (Santa Cruz Biotech., CA), anti-GFP (Molecular Probes, OR), HMB-45 anti-Melanosome antibody (Dako, CA), anti-AU5 (Covance, CA) and anti-HA (Covance, CA). The paraffin-embedded tissue sections were processed as described in immunohistochemistry, and then reacted with anti-GFP (Cell Signaling Technology, MA) and the immunofluorescence reagent as described previously. Image quantification was performed with ImageJ with the MBF ImageJ bundle (http://www.macbiophotonics.ca/imagej/installing_imagej.htm). An automatic threshold was

applied and then the "nucleus counter" plugin was used to draw a region of interest (ROI) around each nucleus in the Hoechst image; the average gray value per ROI was recorded in the corresponding immunostained image (expressed in arbitrary units). Values correspond to the average of approximately 50-100 cells coming from at least 3 different pictures from each experimental condition.

Posphoinositide (PI) turnover assay: HEK293 and OMM1.3 cells were incubated with 1 uCi/mL of [^{3}H]-Inositol for 24 h as previously described (Vaque et al., 2013). After a PBS wash, 1 mL of serum free media was added. After 3 h of incubation, the PLC inhibitor U73122 was added to a final concentration of 1uM. The PI cycle was stopped 1 hr later with LiCl 10 mM final concentration for 20 minutes. The cells were lysed with 5% TCA. The supernatant was transferred to an anion-exchange column (Bio-Rad, AG1-X8, 100-200 mesh) and eluted with a buffer containing 200mM ammonium formate and 100mM formic acid. Radioactivity in each sample were read in a scintillation counter.

***CDKN2A* (*p16^{ink4}*) promoter methylation analysis:** Methylation-specific PCR (MSP) analysis was performed essentially as described in previous studies (Herman et al., 1996).

Luciferase Assays: HEK293 cells were co-transfected with TEAD4-Gal4 (0.5µg/ml), Gal4-luc (0.5µg/ml) and pRLNull (1µg/ml) in 24-well plates overnight to the detection of the luciferase activity, using a Dual-Glo Luciferase Assay Kit (Promega, WI) and a Microtiter plate luminometer (Dynex Tech., VA).

Immunohistochemistry: The following antibodies were used for immunohistochemistry anti-BrdU (Sigma-Aldrich Inc., MO). Unstained 5μm paraffin sections were dewaxed in Safeclear II (Fisher Scientific, PA), hydrated through graded alcohols and distilled water, and washed three times with PBS. Antigens were retrieved using or 10mmol/L citrate buffer boiled in a microwave for 20 min (2 min at 100% power and 18 min at 10% power). The slides were allowed to cool down for 30 min at room temperature, rinsed twice with PBS, incubated in 3% hydrogen peroxide in PBS for 10 min to quench the endogenous peroxidase. The sections were then sequentially washed in distilled water and PBS, incubated in blocking solution (2.5% bovine serum albumin in PBS) for 30 min at room temperature. Excess solution was discarded and the primary antibody (anti-BrdU, Sigma-Aldrich Inc., MO) was applied diluted in blocking solution at 4°C overnight. After washing with PBS, the slides were sequentially incubated with the biotinylated secondary antibody (1:400) (Vector Laboratories, CA) for 30 min and with the avidin-biotin complex, reconstituted according to the instruction of the manufacturer in PBS (Vector Stain Elite, ABC kit) (Vector Laboratories, CA), for 30 min at room temperature. The slides were developed in 3,3-diaminobenzidine (Sigma FASTDAB tablet) (Sigma Chemical, MO) diluted in distilled water under a microscope.

Growth in Soft Agar: Cells were mixed at a concentration of 2,500 cells/0.2 ml of medium, and 0.2% agar (Lonza, MD). The cells in 0.2% agar were plated over 0.2 ml of medium, 1% agar that had been allowed to harden in a 96-well dish. Cells were fed 50μl of medium every 4 days. In the VP treatment assay, VP was added in the medium with final concentration 1μM.

Nuclear and Cytoplasm Extraction: Follow the instructions of NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, CO).

Immunoprecipitation: Cells were lysed with lysis buffer [10mM Tris-Cl (pH 8.0), 150mM NaCl, 1mM EDTA, 0.3% CHAPS, 50mM NaF, 1.5mM Na_3VO_4, protease inhibitor (Thermo Scientific, CO), 1mM DTT, 1mM PMSF], and centrifuged at 16,000g for 10min at 4°C. Supernatants were incubated with first antibody for 2h at 4°C, and protein G or protein A conjugated resin for another 1h. Resins were then washed 3 times with lysis buffer and boiled in SDS-loading buffer.

Actin-AMOT and YAP-AMOT interaction and competition assay: HEK293 expressing flag-YAP were lysed and immunoprecipitated (IP) as described above. After washing, the IP samples were divided into equal samples and incubated with G-actin (0.5 μg/μl) and F-actin (0.5 μg/μl) in lysis buffer, followed by 0.5 hour incubation at room-temperature, washed 3 times with lysis buffer, and boiled in SDS loading buffer. The G-actin and F-actin were generated *in vitro* using the 'Actin Binding Protein Biochem Kit' (Cytoskeleton Inc., CO).

Supplemental References

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