

Supporting Information

Fan et al. 10.1073/pnas.1216462110

SI Materials and Methods

Antibodies and Reagents. All antibodies were obtained from commercial sources, including anti-YAP, anti-GFP, anti-PDK1, anti-Sav1 (Santa Cruz Biotechnology), anti-phospho YAP Ser127, anti-Lats1 (Cell Signaling), anti-Mst1/2 (Bethyl Laboratories), anti-human amphiregulin (R&D Systems), α -tubulin, anti-Flag (Sigma-Aldrich), β -actin (GenScript), and GAPDH (Ambion). The following inhibitors were used: PP2, LY294002, AKT inhibitor V, PDK1 inhibitor II, PD98059, U0126 (EMD Biosciences), BX795 (Santa Cruz Biotechnology), Wortmannin, AKT inhibitor VIII, WP1066, FTI-277 trifluoroacetate, Calphostin C, Gö6983, c-AMP dependent protein kinase inhibitor (IP-20), Rp-adenosine 3',5'-cyclic monophosphorothioate triethylammonium (Sigma-Aldrich), and Iressa (Tocris). Except where indicated otherwise in Fig. S4, all inhibitors were used at a concentration of 10 μ M, except for LY294002 (25 μ M) and BX795 (5 μ M). For treatment, different cell lines were seeded into 24-well plates with 0.3×10^6 cells per well. After 24 h, cells were serum-starved with basal medium supplemented with 1 μ g/mL anti-amphiregulin antibody for 24 h.

Cell Culture, Transfection, and Plasmids. HEK293T and MCF-7 were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Atlanta Biologicals). MCF-10A cells were cultured in DMEM/F12 supplemented with 5% horse serum, 20 ng/mL EGF, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, and 10 μ g/mL insulin (1). A431 (DMEM), HeLa (DMEM/F12), HCT-116 (McCoy's 5a), and HT29 (McCoy's 5a) were grown in their respective media supplemented with 10% FBS (Atlanta Biologicals). HEK293T cells were transfected with Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. MCF-10A or MCF-7 cells were transfected using the Amaxa nucleofector system following the manufacturer's protocol. siRNA were transduced using RNAiMax (Invitrogen) according to the manufacturer's instruction. Transfected cells were harvested at 72 h after transfection for Sav1 knockdown and at different time points for YAP knockdown. For cell proliferation assay, 1×10^5 MCF-10A cells were seeded for siRNA transfection, and after 12 h, the cell culture medium was changed to basal DMEM/F12 containing EGF only (20 ng/mL). EGF was replenished each day. Cells were collected for cell counts every 24 h for up to 108 h after transfection. Oligonucleotides were synthesized by Dharmacon.

To generate the pEGFP-PDK1 construct, PDK1 cDNA was amplified from MCF-10A cell mRNA and subcloned into the BglII-KpnI site of the pEGFP vector (Clontech). The pEGFP-PDK1-RRR472/473/474LLL construct was generated using the QuikChange site-directed mutagenesis kit (Stratagene). All constructs were confirmed by DNA sequencing. HA-Sav1 and

Myc-Mst2 were a kind gift from Dr. Kun-Liang Guan (University of California at San Diego, La Jolla, CA) (2). EGFP-Lats1 and Flag-Lats (Addgene plasmid 19053 and 18971) were a kind gift from Dr. Marius Sudol (Geisinger Clinic, Danville, PA) (3).

Immunoprecipitation and Western Blot Analysis. MCF-10A cells were lysed with hypotonic buffer [10 mM Hepes (pH 7.4), 1 mM EDTA, 150 mM NaCl] supplemented with protease and phosphatase inhibitors. Cell lysates were sheared using a 26G needle and then subjected to low-speed centrifugation (21,130 \times g) for 5 min and then high-speed centrifugation (100,000 \times g) for 1 h. The resulting supernatants were used for immunoprecipitation. HEK293T cells were lysed with Nonidet P-40 buffer [150 mM NaCl, 1.0% Nonidet P-40, 50 mM Tris (pH 8.0)] at 24 h after transfection. Cell lysates were incubated with indicated immunoprecipitation antibodies and Sepharose 4 Fast Flow Protein A/G beads (GE Healthcare). The proteins were resolved on SDS-polyacrylamide gels and transferred to a PVDF membrane (Millipore). HRP-conjugated goat anti-mouse or goat anti-rabbit antibodies were purchased from Jackson ImmunoResearch. Western band intensity was measured by ImageJ. Phos-tag SDS/PAGE was used for separation and detection of large phosphoproteins according to published procedures (4, 5).

ChIP PCR and RT-PCR. In brief, confluent serum-starved MCF-10A cells were treated with or without EGF for 30 min and crosslinked by 1% formaldehyde. Cell nuclei were lysed and sonicated to generate DNA fragments with an average size of 0.5 kb. Anti-YAP or mouse IgG control antibodies were added to the sonicated chromatin fragments for immunoprecipitation. PCR was performed with Phire Hot Start II DNA polymerase (New England Biolabs). The primer sequences in *CTGF* gene promoter amplification were as reported by Zhao et al. (6), with the β -actin gene amplified as an internal control: forward primer, 5'-AAACTGGAACGG-TGAAGGTG-3'; reverse primer, 5'-CTCAAGTTGGGGAC-AAAAA-3'. PCR products were confirmed by sequencing.

To assay induction of *CTGF* mRNA expression, confluent serum-starved MCF-10A cells received control or EGF treatment for 2 h. Total RNA was extracted using the Qiagen RNeasy Kit, followed by cDNA synthesis using SuperScript III Reverse-Transcriptase (Invitrogen). *CTGF* gene or *GAPDH* loading control was PCR-amplified by Phusion high-fidelity PCR polymerase (Thermo Scientific). *CTGF* PCR product size was 504 bp (5' primer, 5'-CTTACCGACTGGAAGACACGTT-3'; 3' primer, 5'-ATGCCATGTCTCCGTACATCTT-3'). *GAPDH* PCR product size was 554 bp (5' primer, 5'-TGGGTGTGAACCATGAGAA-GTA-3'; 3' primer, 5'-TTCGTTGTACATACCAGGAAATG-3').

1. Debnath J, Muthuswamy SK, Brugge JS (2003) Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods* 30(3):256–268.
2. Zhao B, et al. (2007) Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev* 21(21):2747–2761.
3. Oka T, Mazack V, Sudol M (2008) Mst2 and Lats kinases regulate apoptotic function of Yes kinase-associated protein (YAP). *J Biol Chem* 283(41):27534–27546.

4. Kinoshita E, Kinoshita-Kikuta E, Koike T (2009) Separation and detection of large phosphoproteins using Phos-tag SDS-PAGE. *Nat Protoc* 4(10):1513–1521.
5. Zhao B, Li L, Tumaneng K, Wang CY, Guan KL (2010) A coordinated phosphorylation by Lats and CK1 regulates YAP stability through SCF(beta-TRCP). *Genes Dev* 24(1):72–85.
6. Zhao B, et al. (2008) TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev* 22(14):1962–1971.

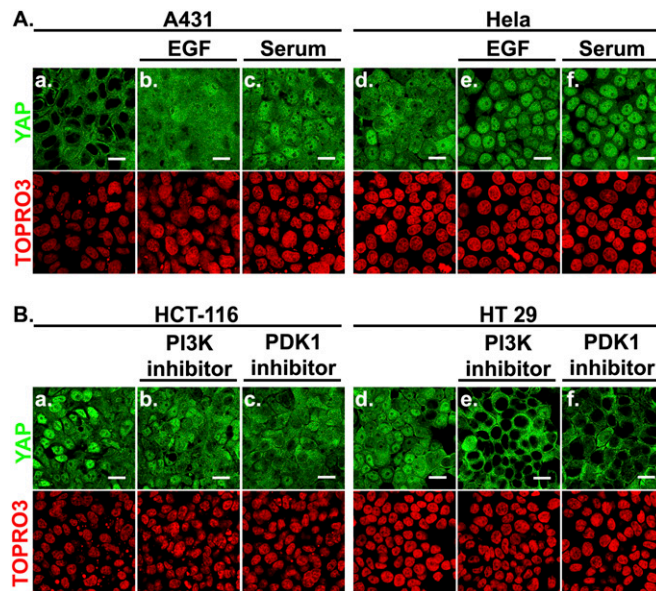


Fig. S1. EGF, serum, and PI3K/PDK1 regulate YAP intracellular localization in various cell types. (A) EGF or serum treatment of confluent serum-starved cells for 30 min induces YAP nuclear accumulation. *a–c*, A431 cells (epidermoid carcinoma). *d–f*, HeLa cells (cervical carcinoma). (B) Inhibition of PI3K (Wortmannin 10 μ M) or PDK1 (BX795 5 μ M) for 2 h induces YAP cytoplasmic retention in tumor cells harboring PI3K mutations. *a–c*, HCT-116 cells (colorectal tumor). *d–f*, HT29 cells (colorectal tumor). All of the images were obtained by confocal immunofluorescence microscopy. Nuclear staining with TOPRO3 is shown below each panel. (Scale bar: 20 μ m.)

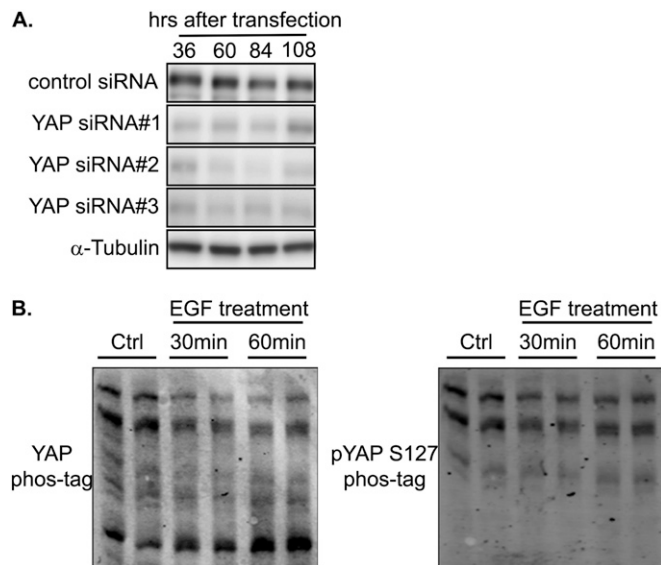


Fig. S2. EGF treatment inhibits Hippo signaling pathway in confluent serum-starved MCF-10A cells. (A) Depletion of YAP by three different siRNAs for the experiment shown in Fig. 1D. The level of YAP expression was determined by Western blot analysis. (B) EGF treatment reduces YAP phosphorylation. MCF-10A cell lysates were resolved on SDS/PAGE gels containing 50 μ M phos-tag conjugated acrylamide to separate the various phosphorylated species. YAP polypeptides were detected by Western blot analysis using anti-YAP (*Left*) or anti-phosphoYAP Ser127 (*Right*) antibodies.

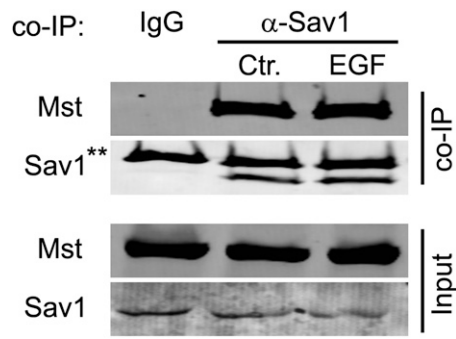


Fig. S5. Mst-Sav1 binding is not affected by EGF treatment in MCF-10A cells. vEGF treatment did not change the binding between Mst and Sav1, as determined by co-IP. Input and co-IP samples were subjected to Western blot analysis with the indicated antibodies. **Antibody heavy chain band.

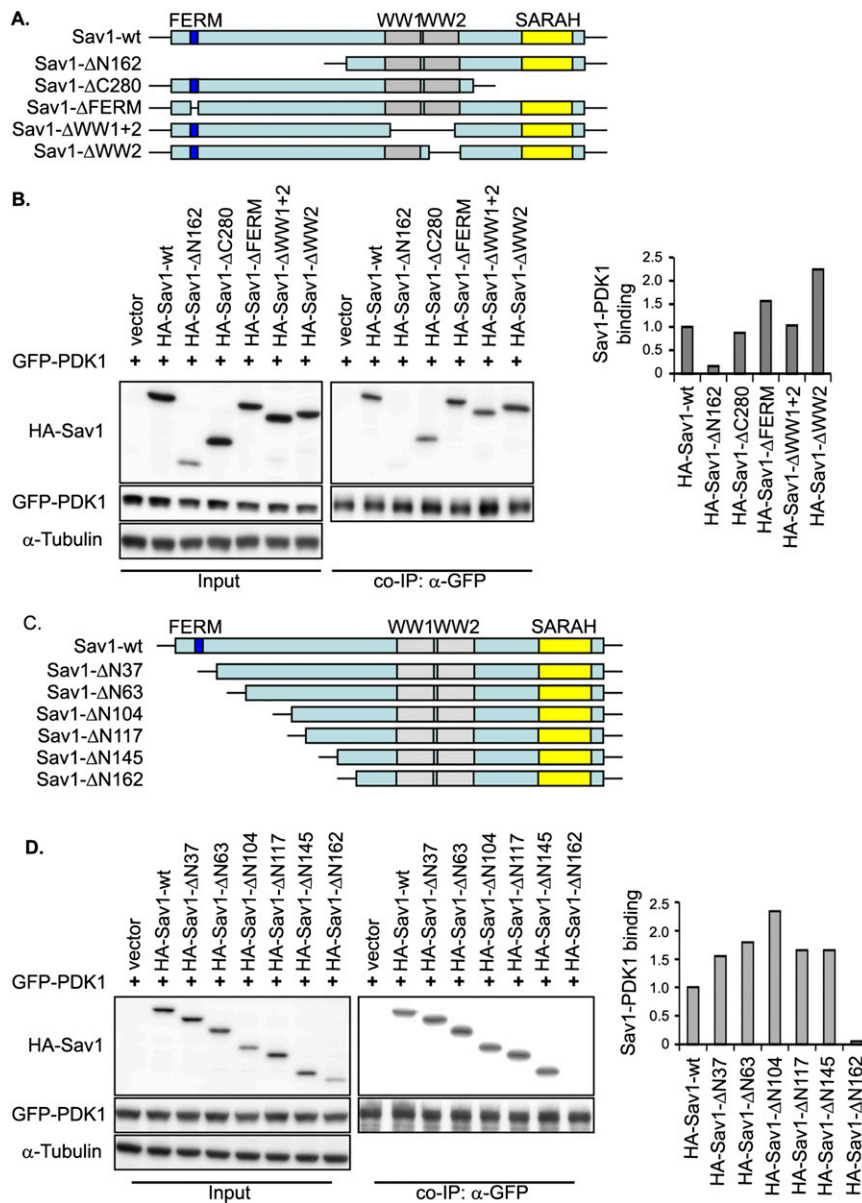


Fig. S7. Residues 145–162 of Sav1 mediate Sav1–PDK1 binding in HEK293T cells. (A) Schematic of WT Sav1 and Sav1 mutants. (B) N terminus of Sav1 mediates Sav1–PDK1 binding by co-IP analysis of exogenously expressed proteins in HEK293T cells. The bar graph shows the relative binding of GFP-PDK1 with WT Sav1 and Sav1 mutants. (C) Schematic of Sav1 N-terminal deletion mutants. (D) Residues 145–162 of Sav1 mediate Sav1–PDK1 binding, as determined by co-IP using exogenous protein expression in HEK293T cells. The bar graph shows the relative binding of GFP-PDK1 with different Sav1 N terminus deletion mutants.

