The Hippo signaling pathway inhibits cell growth and regulates organ size through a kinase cascade that leads to the phosphorylation and nuclear exclusion of the growth-promoting transcriptional coactivator Yes-associated protein (YAP)/Yorkie. It mediates contact inhibition of cell growth downstream of cadherin adhesion molecules and other cell surface proteins. Contact inhibition is often antagonized by mitogenic growth factor signaling. We report an important mechanism for this antagonism, inhibition of Hippo pathway signaling by mitogenic growth factors. EGF treatment of immortalized mammary cells triggers the rapid translocation of YAP into the nucleus along with YAP dephosphorylation, both of which depend on Lats, the terminal kinase in the Hippo pathway. A small-molecule inhibitor screen of downstream effector pathways shows that EGFR receptor inhibits the Hippo pathway through activation of PI3K (PI3K) and phosphoinositide-dependent kinase (PDK1), but independent of AKT activity. The PI3K-PDK1 pathway also mediates YAP nuclear translocation downstream of lysophosphatidic acid and serum as a result of constitutive oncogenic activation of PI3K. PDK1 associates with the core Hippo pathway–kinase complex through the scaffold protein Salvador. The entire Hippo core complex dissociates in response to EGF signaling in a PI3K-PDK1–dependent manner, leading to inactivation of Lats, dephosphorylation of YAP, and YAP nuclear accumulation and transcriptional activation of its target gene, CTGF. These findings show that an important activity of mitogenic signaling pathways is to inactivate the growth-inhibitory Hippo pathway and provide a mechanism for antagonism between contact inhibition and growth factor action.

Results

EGF Treatment Inhibits Hippo Pathway in Confluent MCF-10A Cells. MCF-10A is an immortalized human mammary epithelial cell line that is contact-inhibited at high cell density via the Hippo signaling pathway (5, 10, 11). In serum-starved, contact-inhibited MCF-10A cells, the nuclear effector of the Hippo pathway, YAP, is excluded from the nucleus. However, EGF treatment triggered rapid YAP nuclear accumulation in most cells within 30 min (Fig. 1A). This phenomenon was not unique to MCF10A and mammary cells, but was observed for A431 epidermoid and HeLa cervical carcinoma cell lines as well (Fig. S1A).

To determine whether YAP nuclear translocation is associated with its transcriptional activity, we performed ChIP analysis for promoter-binding activity. YAP is known to associate with the DNA-binding protein TEAD to regulate expression of the connective tissue growth factor (CTGF) gene (12). EGF treatment triggered YAP binding to the CTGF promoter relative to control treatment (Fig. 1B). EGF treatment also increased CTGF mRNA expression compared with control treatment, as determined by RT-PCR (Fig. 1C). To determine whether YAP has a role in EGF-stimulated growth of MCF-10A cells, we knocked down the expression of YAP by siRNA in MCF-10A cells. Three different YAP siRNAs effectively depleted YAP expression for several days compared with control siRNA (Fig. S2A). EGF failed to increase cell numbers in the YAP knockdown group compared with control (Fig. 1D). Taken together, these results show that EGF treatment triggers YAP nuclear accumulation and transcriptional activity, which is critical for EGF-stimulated cell proliferation in MCF-10A cells.

We next examined whether EGF regulates YAP nuclear accumulation through the Hippo pathway. The core Hippo pathway kinase Lats phosphorylates YAP directly at Ser127, which causes YAP cytoplasmic retention (5). EGF treatment reduced overall YAP phosphorylation and YAP phosphorylation at Ser127 in a time-dependent manner in confluent serum-starved MCF-10A cells (Fig. 1E). This reduction was eliminated by treating cells withwortmannin (Fig. 1F). These results indicate that YAP dephosphorylation and nuclear accumulation are mediated through the HIPPO pathway.

The authors declare no conflict of interest.

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Regulation of Hippo pathway by mitogenic growth factors via phosphoinositide 3-kinase and phosphoinositide-dependent kinase-1

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EGF inhibits Hippo Pathway Through PI3K and PDK1, but Independent of AKT Activity.

We used numerous specific pharmacologic inhibitors of its downstream pathways (13) to examine how EGFR affects the Hippo pathway. Confluent, serum-starved MCF-10A cells were pretreated with different inhibitors for 30 min, followed by EGF treatment for another 30 min, and YAP nuclear accumulation was determined by confocal microscopy. Inhibitors of PI3K significantly blocked EGF-mediated YAP nuclear accumulation as effectively as the EGFR inhibitor Iressa (Fig. 2A and Fig. S3B). Inhibitors of the PI3K downstream effector PDK1 were also able to block YAP nuclear accumulation (Fig. 2A and Fig. S3B). Importantly, dose-dependence studies showed that both sets of inhibitors acted at low concentrations expected for specific effects on these enzymes (Fig. S4). In contrast, inhibitors of another major kinase downstream of PI3K, AKT, had no effect on YAP nuclear accumulation (Fig. 2A). None of the other inhibitors, including Src family kinase inhibitor, MEK inhibitors, Jak2 inhibitor, PKC inhibitors, Ras/farnesyltransferase inhibitor, and PKA inhibitors had any effect on EGF-mediated YAP nuclear accumulation (Fig. S3). Moreover, both the PI3K inhibitor and the PDK1 inhibitor blocked the reduction in YAP Ser127 phosphorylation caused by EGF treatment (Fig. 2B). These results demonstrate that EGFR regulates the Hippo pathway through PI3K-PDK1 activity.

Because PI3K can be activated by growth factors other than EGF, we tested whether this PI3K-PDK1-mediated regulation of the Hippo pathway is specific to EGFR signaling. Lysoosphatidic acid (LPA) or horse serum, which can activate PI3K signaling independent of EGFR signaling (14), both caused YAP nuclear accumulation within 30 min of treatment of MCF-10A cells (Fig. S4). Importantly, PI3K and PDK1 inhibitors blocked YAP nuclear accumulation caused by LPA or serum treatment.
Pretreatment of the cells by the EGFR inhibitor Iressa had no effect on YAP nuclear accumulation induced by LPA or serum, ruling out an indirect effect via EGFR (Fig. 3A) (15). A different human mammary cell line, MCF-7, harbors a constitutive active ES545K mutation in the PI3K gene, PIK3CA (16). In contrast to MCF-10A cells, YAP remained in the nucleus in confluent serum-starved MCF-7 cells even after serum starvation (Fig. 3B). Importantly, the PI3K or PDK1 inhibitors caused YAP to accumulate in the cytoplasm of MCF-7 cells, demonstrating that constitutive activation of PI3K and PDK1 activity was responsible for YAP nuclear accumulation in confluent, serum-starved MCF-7 cells. Two colon cancer cell lines, HCT-116 and HT29, also carry activating mutations in the PIK3CA gene (17), and both the PI3K and PDK1 inhibitors similarly caused accumulation of YAP in the cytoplasm of both cell lines (Fig. S1B). These data suggest that many, if not all, extracellular stimuli or oncogenic mutations that activate PI3K-PDK1 signaling will inhibit Hippo pathway signaling and trigger YAP nuclear accumulation.

**PDK1 Forms a Complex with Hippo Pathway Molecules in Confluent Serum-Starved Cells, and EGFR Treatment Dissociates the PDK1-Hippo Complex and the Entire Hippo Pathway-Kinase Complex.** Components of the Hippo kinase cascade, including Mst, Sav1, and Lats, form a complex (2, 18–20). These proteins coimmunoprecipitate with endogenous PDK1 in confluent, serum-starved cells (Fig. 4A), demonstrating that PDK1 is in the same complex as Hippo pathway kinases. EGF treatment for 30 min disrupted the PDK1 interaction with these Hippo pathway components, as evidenced by the loss of Lats1, Mst, or Sav1 in the PDK1 immunoprecipitates. Importantly, the PI3K inhibitor and PDK1 inhibitor blocked the effect of EGF on dissociation of PDK1 from the Hippo complex. We also performed a reciprocal coimmunoprecipitation (co-IP) using anti-Lats1 antibody and found that PDK1, as well as Mst and Sav1, coimmunoprecipitated with endogenous Lats1 in serum-starved cells. The interaction of Lats with all of these components was disrupted after 30 min of EGF treatment (Fig. 4B), and pretreatment with the P3K inhibitor blocked the effect of EGF on complex dissociation. In contrast, the Sav1–Mst interaction assessed by co-IP was not affected by EGF treatment (Fig. S5). These co-IP experiments identify PDK1 as a component of the Hippo pathway complex. They also show that brief EGF treatment causes dissociation of the complex in a way that would be expected to disrupt the kinase cascade owing to the disruption of Lats from Mst and Sav. Finally, these data show that EGF-induced complex dissociation depends on the activities of PI3K and PDK1, suggesting that PDK1 regulates dissociation.

To better understand how PDK1 interacts with the Hippo complex, we studied the PDK1–Lats interaction by deletion mutagenesis and co-IP analysis (Fig. S6A). Deletion of the Lats PPxY motif completely disrupted the Lats–PDK1 association in HEK293T cells (Fig. S6B). However, the PPxY motif binds to molecules containing the WW domain, which is absent in PDK1. Thus, Lats may associate with PDK1 indirectly via the WW domain-containing molecule Sav1. Indeed, we found that overexpression of Sav1 increased the binding between PDK1 and Lats by 2.6-fold compared with vector control (Fig. S4). Sav1 binds to Mst through their SARAH domains (18), and we found that Sav1 increased the PDK1–Mst association in a SARAH domain-dependent manner (Fig. S6C), suggesting that Sav1 mediates the association between PDK1 and Mst.

To further test the idea that Sav1 is the central molecule that mediates the interaction of PDK1 with other Hippo components, we knocked down Sav1 by siRNA in MCF-10A cells. Although PDK1–Lats1 and PDK1–Mst associations were detected in control siRNA-transfected cells, loss of Sav1 abolished the PDK1–Lats1 and PDK1–Mst interactions.
mediated knocked down of Sav1 used in Statistical significance was calculated using the Student t test. (B) siRNA-mediated knocked down of Sav1 used in C. in MCF-10A cells. The bar graph shows Sav1 levels normalized to β-actin (n = 5). Error bar represents mean ± SEM. Statistical significance was calculated using the Student t test. (C) Sav1 mediates PDK1–Hippo complex binding in MCF-10A cells. Lysates from control siRNA or Sav1 siRNA-treated cells was incubated with anti-PDK1 antibody. Co-IP and input samples were subjected to Western blot analysis with indicated antibodies.

and PDK1–Mst associations (Fig. 5B and C). Analysis of the interactions of a series of Sav1 deletion mutants with PDK1 in HEK293T cells showed that amino acid residues 145–162 of Sav1 molecule are critical for the PDK1–Sav1 interaction (Fig. S7). Thus, Sav1 is the central molecule that mediates the interaction of PDK1 with the Hippo pathway.

**PDK1 Pleckstrin Homology Domain Is Important for EGF-Mediated Hippo inhibition.** PDK1 comprises an N-terminal kinase domain and a C-terminal pleckstrin homology (PH) domain, and is recruited to the plasma membrane on growth factor stimulation through PH domain binding to PtdIns(3,4,5)P3, which leads to PDK1 activation (21–23). To determine whether PDK1 PH domain-mediated membrane recruitment and activation is important for the function of PDK1 in EGF regulation of the Hippo pathway, we generated a PDK1 PH domain mutant, PDK1-RRR472/473/474LLL (PDK1-R472/3/4L), which cannot bind PtdIns(3,4,5)P3 and shows impaired kinase activity (21, 23, 24). Expression of the PDK1-R472/3/4L in confluent serum-starved MCF-10A cells reduced YAP nuclear accumulation in response to EGF treatment compared with WT PDK1 (Fig. 6). PDK1-R472/3/4L was expressed at only 10% of the level of WT PDK1 in MCF-10A cells (Fig. S8A), consistent with observations in a previous study (24), which explains its only partial effect on EGF-stimulated YAP nuclear accumulation.

We also performed this experiment in MCF-7 cells, and found that PDK1-R472/3/4L significantly decreased YAP nuclear staining compared with WT PDK1 (Fig. 6C). We used co-IP to examine how the PH domain influences the interaction of PDK1 with the Hippo complex. Although PDK1-R472/3/4L was expressed at only 20% of the level of WT PDK1 in HEK293T cells, more Sav1 bound to PDK1-R472/3/4L than to WT PDK1 (Fig. 6D). We obtained similar results for the PDK1-R472/3/4L mutant and Lats1 association (Fig. S8B). These findings suggest that PDK1 affects the Hippo pathway complex as a result of membrane recruitment on EGF treatment.

We propose a model to explain our findings (Fig. 7). In serum-starved confluent cells, PDK1 remains in the cytoplasm and forms a complex with active Hippo pathway kinases that phosphorylate YAP to retain it in the cytoplasm. When growth factors are added, PDK1 is recruited onto plasma membrane, leading to Hippo complex dissociation. Complex dissociation inactivates Lats, leading to YAP dephosphorylation and nuclear accumulation.
Hippo complex dissociates, preventing regulation of Lats by Mst, which (EGF, LPA, or serum), PDK1 is recruited on to the membrane, and the PDK1-in the cytoplasm, and cell growth is arrested. In the presence of growth factors Hippo pathway proteins (Lats, Mst, and Sav1), and the Hippo pathway is active. Model for growth factor regulation of the Hippo pathway. In con-

Discussion

Our findings demonstrate that mitogenic growth factor signaling rapidly inactivates the Hippo signaling pathway in confluent contact-inhibited cells. EGF signaling, as well as signaling stimulated by serum and LPA, caused rapid nuclear accumulation of the YAP transcriptional activator. Although nuclear accumulation of YAP is also known to be regulated by Hippo pathway-independent mechanisms (25, 26), we used several other criteria to establish the Hippo pathway itself as an important target of EGF signaling. We found that dephosphorylation of YAP at Ser127, a target for Lats, a key component of the Hippo kinase cascade, is associated with its nuclear accumulation. Moreover, YAP nuclear accumulation in response to EGF depends in part on Lats. Finally, we observed rapid dissociation of the core Hippo pathway complex of kinases and accessory/scaffolding proteins in response to EGF signaling, demonstrating that the core Hippo pathway complex is a target of EGF signaling action.

Extracellular mediators that regulate the Hippo pathway include the Drosophila/Fat system (27) and polarity proteins, such as crumbs (28–30) in Drosophila. Cell contact is known to activate Hippo signaling, and we previously identified cadherin-mediated contacts as responsible (10); evidence for roles of other junction-associated proteins has been reported as well (26, 31, 32). Our current findings show that growth factor receptors are upstream negative regulators of the Hippo pathway. Similarly, in Drosophila, insulin-like growth factor (IGF) regulates growth through Yorkie/YAP (33). Soluble factors in serum have recently been reported to act through G protein-coupled receptors to either activate or inhibit the Hippo pathway, although the mechanisms were not identified (34). Clearly, the factors controlling the Hippo pathway activity are numerous and varied.

We discovered a common mechanism by which various types of mitogenic pathways inactivate the Hippo pathway. Although we used EGF as a model, we found that both serum and LPA, which works through a G protein-coupled receptor, stimulated rapid nuclear accumulation of YAP. Indeed, our discovery that this activity is mediated by PI3K and PDK1 suggests that many, if not all, of the various signaling pathways that activate PI3K signaling will inhibit the Hippo pathway. We even found that a constitutively active oncogenic mutation in PI3K drives YAP nuclear localization in mammalian tumor cells. Although the PI3K inhibitor Wortmannin was not found to affect YAP phosphorylation in response to FBS (34), the effects of Wortmannin and other PI3K and PDK1 inhibitors in our system were very robust and reproducible. In agreement with our findings, IGF regulation of Hippo signaling in Drosophila was found to be mediated by PI3K and PDK1 (33).

Despite the key roles of PI3K and PDK1 in growth factor regulation of Hippo signaling, AKT, the major effector kinase that typically acts downstream of these proteins, does not appear to have a role in Hippo pathway regulation in MCF-10A cells. This suggests that alternative effectors are important. PDK1 is known to have other substrates (35), but our findings implicate PDK1 directly in control of the Hippo pathway. The protein target phosphorylated by PDK1 important for control of Hippo signaling is not yet clear; we have not been able to identify known components of the Hippo complex as direct targets. Nonetheless, our finding that PDK1 forms a protein complex with the core Hippo complex through its interaction with Sav1 indicates that the Hippo pathway is an important target of PDK1 activity.

The mechanism by which growth factor-induced PI3K-PDK1 activity inhibits the Hippo pathway appears to be related to dissociation of the core Hippo complex containing Mst, Sav1, Lats, and PDK1. Complex dissociation is rapid, occurring in a similar time frame as nuclear accumulation and dephosphorylation of YAP. Like YAP nuclear accumulation, hippo complex dissociation depends on the activities of PI3K and PDK1. Formation of this complex is well known to be critical for Hippo pathway activity, given that the complex and the scaffolding protein Sav1 are required for the phosphorylation of Lats and Mob by Hippo/Mst (2, 19, 20, 36). Thus, complex dissociation is expected to result in a loss of Lats activation, leading to dephosphorylation of YAP. Although the formation of the core complex is known to be important for Hippo activity, our findings demonstrate that active dissociation of the complex by regulatory factors plays a role in control of Hippo pathway activity.

Exactly how PI3K and PDK1 activities control the state of the core Hippo complex is not yet clear, but likely involves PH domain-mediated recruitment of PDK1 to the plasma membrane by phosphoinositide products of PI3K. Because a mutant form of PI3K lacking the PH domain associates more abundantly with the Hippo complex, membrane recruitment of PDK1 may be involved in the control of complex dissociation.

Inhibition of the growth-inhibitory Hippo pathway by mitogenic growth factor signaling raises interesting ideas about integration of various types of growth control mechanisms. Inactivation of the Hippo pathway may be an important requirement for growth factors to trigger proliferation. Indeed, we found that YAP is required for EGF stimulation of cell proliferation in MCF-10A cells. This is surprising and important, because it suggests that, for this cell type at least, the other branches of the EGF pathway may be insensitive on their own to stimulate growth. Similarly, YAP and Yorkie were found to be required for IGF-stimulated growth in Drosophila (33), and serum GPR stimulated growth in HEK293A cells (34). Moreover, these pathways likely create important autocrine and other feedback loops via transcriptional targets of YAP, including CTGF, amphiregulin, and upstream components of the IGF pathway (12, 33, 37, 38). Amphiregulin seems to inhibit the Hippo pathway in MCF-10A cells, as demonstrated by the need to add neutralizing antibodies for effective serum starvation. The interactions between these pathways may be important for understanding tissue growth in vivo.

 Oncogenic activation of RTKs, as well as PI3K, are known to contribute to tumor formation (39–41). Our findings raise the possibility that nuclear YAP is an important downstream effector of these pathways that contributes to tumor growth and has been shown to have oncogenic activity when activated by mutations.
that block its regulation by the upstream Hippo pathway (4). It will be important to investigate this relationship between RTK and Hippo pathways in tumorigenesis experimentally in animals and in human tumors.

Materials and Methods

Cell Culture and Treatment. Confluent MCF-10A cells were serum-starved with basal medium supplemented with 1 μg/mL anti–amphiregulin-neutralizing antibody for 24 h. Cells were pretreated with indicated inhibitors for 30 min, and then treated with 20 ng/mL EGF (PeproTech), 25 μM Iyosphosphatidic acid (LPA; Sigma-Aldrich), or 5% horse serum in basal DMEM/F12 medium (Invitrogen). Other cells were cultured as described previously (10). Antibodies, reagents, plasmids, and transfections are described in detail in SI Materials and Methods.

Immunofluorescence Staining and Confocal Microscopy. Cells were fixed with 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100.


Supporting Information

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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-phosphoYAP 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, wortmannin ET, and N2 MCF-10A cell RNA polymerase inhibitor C, Go6983, c-AMP dependent protein kinase inhibitor (IP-20), Rp-adenosine 3’-5’,cyclic monophosphoroethioate triethylammonium (Sigma-Aldrich), and Iressa (Tocris). Except where indicated otherwise in Fig. S4, all inhibitors were used at a concentration of 10 μM, except for LY294002 (25 μM) and BX795 (5 μM). 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 μg/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 (Atlanta Biologicals), 10% FBS (Atlanta Biologicals), and 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 transfected 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 Dharmaco.

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-RRR472/473/474/LLL 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). EGF-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 × g) for 5 min and then high-speed centrifugation (100,000 × g) for 1 h. The resulting supernatants were used for immunoprecipitation. HEK293T cells were lysed with Nonidet P-40 buffer [150 mM NaCl, 1% 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’-AAACTGCCAGGTTAGGTTG-3’; reverse primer, 5’-CTCAAGTGGGCGACAAAAA-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 Qia-gen 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’-CTTCCCGAGTTGAGGATCGT-3‘; 3’ primer, 5’-ATGCCATGTCCTCCTGATCCTT-3’). *GAPDH* PCR product size was 554 bp (5’ primer, 5’-TGGTGTGAACTGACATTAGAATA-3‘; 3’ primer, 5’-TTCTGGTGCTATACCGAAGG-3’).

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 μM) or PDK1 (BX795 5 μM) 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 μm.)

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 μM 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. S3. EGF treatment inhibits the Hippo pathway through the PI3K-PDK1 pathway, independent of AKT activity. (A) In the same experiment shown in Fig. 2, inhibitor screening of the EGF signaling pathway in MCF-10A cells was done by confocal microscopy of YAP nuclear accumulation. Cells in c–k received a 30-min inhibitor pretreatment, followed by a 30-min EGF treatment. a, no treatment; b, EGF treatment; c, PP2; d, PD98059; e, U0126; f, WP1066; g, G06983; h, Calphostin C; i, FTI-277; j, Rp-CAMPS; k, IP-20. (Scale bar: 20 μm.) (B) Quantification of data shown in A and Fig. 2A. The bar graph shows the percentage of cells with a nuclear, a cytoplasmic, or both a nuclear and cytoplasmic staining pattern. The percentage of cells with nuclear staining was compared in the indicated groups. Statistical significance was calculated using the two-sided Fisher exact test. ***P < 0.0001.
Fig. S4. Dose-dependence of PI3K and PDK1 inhibitors used to inhibit EGF-induced YAP nuclear accumulation. (A) Confluent MCF-10A cells were serum-starved for 24 h, pretreated for 30 min with the indicated inhibitor at the doses shown, followed by a 30-min EGF treatment. a, no treatment; b, 30 min EGF treatment alone; c-f, Wortmannin (PI3K inhibitor); g-i, LY294002 (PI3K inhibitor); j-m, PDK1 inhibitor II; n-q, BX795 (PDK1 inhibitor). YAP localization was determined by confocal microscopy. Nuclear staining with TOPRO3 is shown below each panel. (Scale bar: 20 μm.) (B) Quantification of data shown in A. The bar graph shows the percentage of cells with a nuclear, a cytoplasmic, or both a nuclear and cytoplasmic staining pattern. The percentage of cells with nuclear staining was compared with that in the EGF treatment group. Statistical significance was calculated using the two-sided Fisher exact test. *P < 0.0001.
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. S6. PDK1 interacts with Lats1 and Mst through scaffold protein Sav1 in HEK293T cells. (A) Schema of WT Lats1 and Lats1 mutants. Lats contains three domains important for its function in the Hippo pathway: (i) kinase domain, which is critical to phosphorylate YAP; (ii) two PPxY motifs, which mediate Lats interaction with proteins containing the WW domain (e.g., YAP and Sav1); and (iii) a Mob-binding domain. A series of deletion or truncation mutants were made based on these different domains. (B) PPxY motifs of Lats1 are critical for PDK1–Lats1 interaction, as determined by co-IP using exogenous protein expression in HEK293T cells. The bar graph shows the relative binding between Flag-Lats1 and GFP-PDK1. *Nonspecific bands. (C) The Mst2–PDK1 association depends on the Sav1 SARAH domain as determined by co-IP using exogenous protein expression in HEK293T cells. The schematic shows WT Sav1 and Sav1-ΔC280 mutant without the SARAH domain.
**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.
Fig. S8. Properties of PDK1-R472/3/4L mutant. (A) Expression of PDK1-R472/3/4L is lower than that of WT PDK1 in MCF-10A cells. GFP-PDK1-wt or GFP-PDK1-R472/3/4L was transfected into MCF-10A cells by electroporation. At 60 h after transfection, MCF-10A cells were collected, and whole-cell lysates were subjected to Western blot analysis with anti-PDK1 antibody. (B) PDK1 PH domain defective mutant binds to Lats1 more abundantly than WT PDK1 in HEK293T cells. Exogenous proteins were coexpressed in HEK293T cells. PDK1 and Lats1 interactions were determined by co-IP and Western blot analysis. The graph quantifies Western blot analysis results. Statistical significance was calculated using the Student t test. The error bar represents mean ± SEM; n = 3.