

Identification of Serum-Derived Sphingosine-1-Phosphate as a Small Molecule Regulator of YAP

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SUMMARY

Hippo signaling represents a tumor suppressor pathway that regulates organ size and tumorigenesis through phosphorylation and inhibition of the transcription coactivator YAP. Here, we show that serum deprivation dramatically induces YAP Ser127 phosphorylation and cytoplasmic retention, independent of cell-cell contact. Through chemical isolation and activity profiling, we identified serum-derived sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) as small molecule activators of YAP. S1P induces YAP nuclear localization through S1P₂ receptor, Rho GTPase activation, and F-actin polymerization, independent of the core Hippo pathway kinases. Bioinformatics studies also showed that S1P stimulation induces YAP target gene expression in mouse liver and human embryonic stem cells. These results revealed potent small molecule regulators of YAP and suggest that S1P and LPA might modulate cell proliferation and tumorigenesis through YAP activation.

INTRODUCTION

Organ size regulation is a highly orchestrated process involving multiple signaling networks that respond to physiological cues. Initially discovered in *Drosophila*, Hippo signaling is an emerging tumor suppressor pathway that plays key roles in normal physiology and tumorigenesis through the regulation of cellular proliferation and survival (Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Tapon et al., 2002; Udan et al., 2003; Wu et al., 2003). The core components of Hippo signaling are well conserved in *Drosophila* and vertebrate, including Ste20-like kinases MST1/2 (Hippo/Hpo in *Drosophila*), adaptor protein SAV1 (Salvador/sav in *Drosophila*), NDR family kinases Lats1/2 (Warts/Wts in *Drosophila*), and transcription coactivators YAP and TAZ (Yorkie/Yki in *Drosophila*) (Harvey and Tapon, 2007). The signal transduction involves a core kinase cascade, in which SAV1 facilitates MST1/2 kinases to phosphorylate and activate

Lats1/2. Subsequently, Lats1/2 phosphorylate YAP and TAZ at the consensus HXRXXS motifs, including Ser127 of YAP and Ser89 of TAZ (Zhao et al., 2007). These phosphorylation events are inhibitory to the transcription coactivator function of YAP and TAZ. Phosphorylated YAP and TAZ are sequestered in the cytoplasm by binding to 14-3-3 protein (Pan, 2010). Inactivation of the upstream kinases leads to the accumulation of YAP and TAZ in the nuclei and promotes their binding to other transcription factors, including TEA domain transcription factors (TEAD) to regulate target genes expression (Chan et al., 2009; Goulev et al., 2008; Ota and Sasaki, 2008; Wu et al., 2008; Zhang et al., 2008, 2009; Zhao et al., 2008).

Previous work has shown that cell-cell contact can activate mammalian Hippo signaling, leading to MST1/2 and Lats1/2 kinases-mediated inhibition of YAP and TAZ. Extracellular matrix (ECM) stiffness can modulate YAP and TAZ through Rho GTPase and cytoskeletal rearrangement, independent of the core Hippo kinases (Dupont et al., 2011; Zhao et al., 2011b). Enhanced F-actin polymerization induces organ overgrowth in *Drosophila* through activation of Yorkie (Fernández et al., 2011; Sansores-Garcia et al., 2011; Wada et al., 2011). Cellular tight junction (TJ) protein angiomin and adherens junction (AJ) protein α -catenin can directly bind to YAP and modulate its localization and Ser127 phosphorylation (Chan et al., 2011; Schlegelmilch et al., 2011; Zhao et al., 2011a). In addition, Fat and Dachsous have been identified as receptor and ligand regulating *Drosophila* Hippo signaling (Bennett and Harvey, 2006; Cho et al., 2006; Rogulja et al., 2008; Silva et al., 2006; Tyler and Baker, 2007; Willecke et al., 2006, 2008). However, upstream signals that affect YAP in mammals require further investigation.

RESULTS

Serum Deprivation Induces YAP Cytoplasmic Retention

To identify regulators of YAP, we utilized an imaging-based YAP nuclear localization assay that recapitulated cell-cell contact-mediated YAP regulation: culturing HEK293A cells (an adherent subclone of HEK293 cells) at low density (~50% confluency) showed predominant YAP nuclear localization, whereas culturing cells at high density (~100% confluency) led to Hippo pathway activation and predominant YAP cytoplasmic localization (Zhao et al., 2007) (Figure S1 available online). The

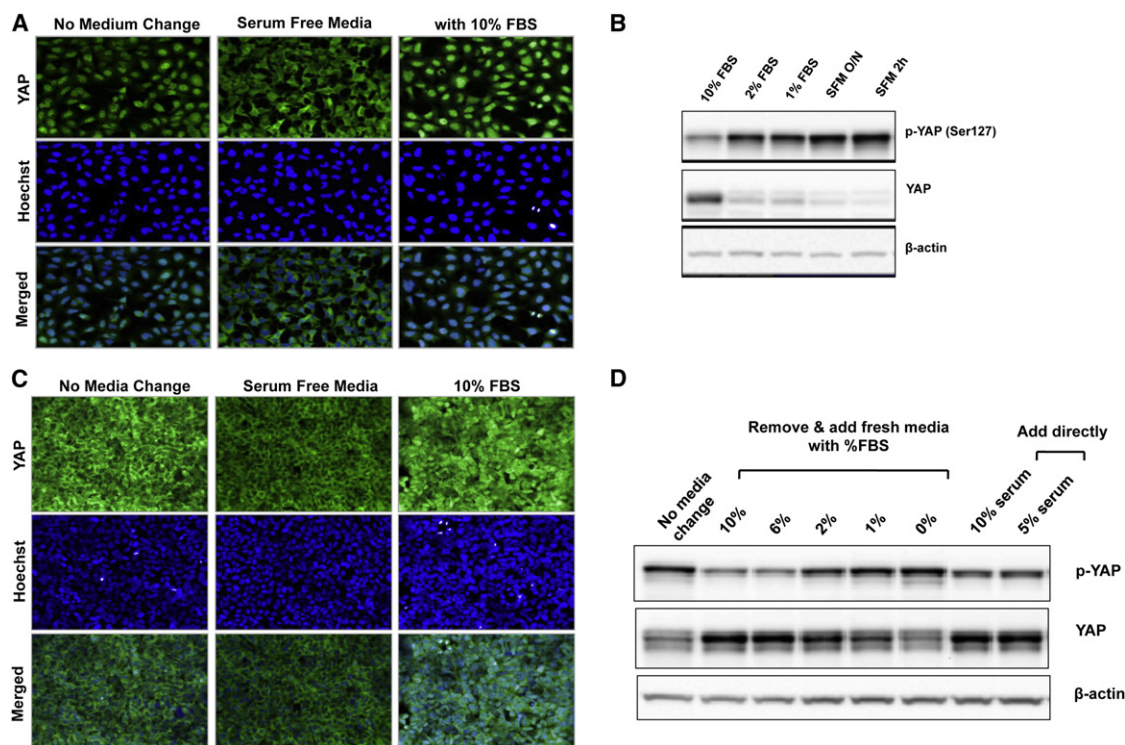


Figure 1. Serum Factor Induces YAP Nuclear Localization

(A) Serum deprivation induces YAP cytoplasmic localization. Normal culture media (with 10% FBS) in low-density HEK293A cells was replenished with serum-free media (SFM) or fresh media containing 10% FBS for 2 hr. Cells are fixed and immunostained for YAP (green) and nuclei are stained with Hoechst (blue).

(B) Serum deprivation induces YAP Ser127 phosphorylation. Cells were treated with media containing 10% FBS, 2% FBS, 1% FBS, or serum-free medium for 12 hr (SFM O/N), or serum-free media for 2 hr (SFM 2 hr). The total YAP protein level inversely correlates with p-YAP (S127) level, consistent with the previous reports that YAP phosphorylation primes its degradation.

(C) HEK293A cells cultured at high density (~100% confluency) in normal media (DMEM + 10% FBS) exhibit high YAP cytoplasmic localization. After replacing the media with serum-free DMEM (SFM) for 2 hr, YAP remains predominantly in the cytoplasm. After replacing the media with fresh DMEM + 10% FBS for 2 hr, high levels of nuclear YAP are observed, indicating that serum factors can override contact-dependent YAP regulation.

(D) Western blot analysis of p-YAP (Ser127) in HEK293A cells cultured at high density. Culture media were replaced with DMEM supplemented with different concentrations of FBS (lanes 2 to 6). Additional FBS was added directly in cells without media change in lanes 7 and 8 (data are represented as mean \pm SEM, $n = 3$). See also Figures S1, S2, S3, and S4.

Pearson's correlation coefficient between YAP-positive area and cell nuclei was used to quantify YAP nuclear localization (see the [Supplemental Experimental Procedures](#)). During assay optimization, we serendipitously observed that YAP cytoplasmic retention and Ser127 phosphorylation could be dramatically induced by decreasing the concentration of fetal bovine serum (FBS) in culture medium. This effect was independent of cell-cell contact and could be induced within 2 hr upon serum deprivation (Figures 1A and 1B; Figures S2A and S2B). Furthermore, adding fresh serum to confluent HEK293A cells could induce YAP Ser127 dephosphorylation and nuclear localization, overriding cell-cell contact-mediated YAP inhibition (Figures 1C and 1D). We observed a similar effect in additional cell lines (HaCaT, NIH 3T3, OVCAR-8, and RT-4) although less dramatic as HEK293A cells and with sera from multiple species (goat, rabbit, horse, and human), suggesting that such an effect could be a conserved signaling event (Figures S2C and S3). Therefore, we hypothesized that some unknown factor(s) in serum might be novel ligand(s) regulating YAP. In addition, we found that MST1/2 and Lats1/2 kinases are dispensable for YAP Ser127 phosphorylation induced by serum deprivation, as siRNA-mediated

silencing of these upstream regulators does not block the effects (Figure S4). The total YAP levels inversely correlate with p-YAP levels, consistent with the previous report that phosphorylation of YAP promotes its degradation (Zhao et al., 2010).

Chemical Isolation, Purification, and Activity-Guided Profiling of Serum Factors

Serum is a complex mixture of growth factors, nutrients, lipids, and metabolites. To identify the active serum factor(s), we utilized an activity-guided isolation methodology employing the imaging-based YAP translocation assay. Specifically, we replaced the cell culture media (10% FBS) of low-density HEK293A cells with serum-free media (SFM) that had been supplemented with various serum-derived fractions. After 2 hr of incubation, we examined the YAP subcellular localization status to test whether any serum-derived sample could restore YAP nuclear localization.

We initially observed that the serum-derived activity survived heat inactivation (60°C, 30 min), treatment that usually inactivates complement factors (Figure S5). In order to refine the serum, we utilized methanol precipitation followed by

partitioning of the resulting supernatant against hexanes, to localize the majority of the activity to an enriched aqueous fraction (Want et al., 2006) (Figure S5). Separation of the active serum extract by reversed phase high-performance liquid chromatography (HPLC) (C₁₈) and subsequent activity profiling led to the identification of two active regions within the 40% and 60% CH₃CN in H₂O steps of the elution gradient (Figure S6). To reveal the components of these fractions, we first conducted protein mass spectrometry on the two most active fractions (F029 and F037) and an inactive fraction (F021) to determine if any proteins were selectively present in the active fractions. Our analyses revealed the presence of multiple proteins, including apolipoprotein (Apo) A1, A2, and E. Interestingly, the number of identifiable spectra corresponding to ApoA2 was greatest in the active fractions and lowest in the inactive fraction (Table S1).

Lipids Associated with Lipoproteins Are the Active Components

We tested purified, lipid-stripped human and bovine ApoA2 and found that the protein itself was inactive. We then tested purified lipid-bound human HDL and low-density lipoprotein (LDL), which contain apolipoproteins (Schonfeld et al., 1978), and found that both induced YAP nuclear translocation (Figure S7A). Consistently, we also observed that boiling (95°C, 30 min) of FBS, which usually denatures serum protein factors, did not affect the activity to induce YAP nuclear localization (Figure S5). In addition, charcoal/Dextran-treated, delipidated FBS lost the activity, suggesting that serum lipids might be the active component(s) (Figures S2A and 3B). Taken together, we hypothesized that the overall serum activity was likely lipid based and the specific activity observed in F029 and F037 might be derived from free lipids that coeluted with ApoA2, or residual lipid-bound ApoA2, resulted from incomplete delipidation during the purification process.

To confirm the lipid activity, we then extracted fraction F037 and human HDL with butanol and tested the resulting organic and aqueous partitions. In each of the extractions, the YAP nuclear translocation activity was present in the organic (lipid containing) partition, whereas the aqueous partitions (protein) were inactive (Figure S7). However, attempts to further purify and profile the lipids in these fractions were not successful because of the limited amount of active fraction available.

Sphingosine-1-Phosphate Is Located in the Active Fractions

We attempted to crudely separate different lipids contained within HDL, F029, and F037, utilizing a modified Bligh and Dyer extraction system described previously that yielded three partitions (Kimura et al., 2001): (1) fatty acids, neutral lipids, and phospholipids; (2) charged lipids soluble in aqueous solution under alkaline conditions (such as sphingosine-1-phosphate, S1P); and (3) substances soluble in an aqueous solution (e.g., proteins). In each case, the majority of the activity present in the parent sample was located in partition b (Figure 2A). Partition b of the most active fraction (F037) displayed dose-responsive activity with an approximate EC₅₀ of 1.66 µg/ml (Figure 2B). These results indicate that lipids soluble under alkaline conditions, such as S1P and sphingolipids, might be the active factor(s).

Previous reports have also shown that S1P indeed induces the expression of *Cyr61* and *CTGF*, both direct target genes of YAP (Chowdhury and Chaqour, 2004; Li et al., 2008; Young et al., 2009), suggesting that S1P might activate YAP transcriptional activity. S1P is a well-described signaling lipid bound to HDL, known to modulate diverse cellular functions, including cell proliferation, migration, and differentiation (Argraves and Argraves, 2007; Pyne and Pyne, 2010; Spiegel and Milstien, 2003). The serum concentration of S1P ranges from ~200 nM up to low micromolar, with ~65% bound to lipoproteins (Murata et al., 2000). In addition, it is also known that S1P can activate Rho GTPase, a possible regulator of YAP as described recently (Dupont et al., 2011). Taken together, we hypothesized that S1P might be one of many serum factors present in the active fractions that regulate YAP.

We then compared the extracts to the synthetic *D-erythro*-S1P by high-resolution liquid chromatography/mass spectrometry (LC/MS) and confirmed that S1P was present in the active samples (partition b) and absent in the inactive samples (Figures 2C and 2D). The fragmentation pattern of S1P from F37b also matched with the synthetic standard (Figure S8).

S1P, Dihydro-S1P, and Lysophosphatidic Acid Activates YAP

To confirm that S1P dose-dependently induces YAP nuclear localization, we tested a purified and synthetic *D-erythro* form of S1P, dihydro-S1P (dhS1P), and lysophosphatidic acid (LPA), lipids that activate related receptors (Choi et al., 2010; Rosen and Goetzl, 2005). As shown in Figure 3A and Figures S9 and S10, all three compounds induced YAP nuclear localization with S1P being the most potent ligand (S1P: EC₅₀ = 17 nM; dhS1P: EC₅₀ = 146 nM; LPA: EC₅₀ = 316 nM). Another related lipid, ceramide-1-phosphate (C1P) is inactive (Figure S9). Sphingosine-1-phosphate also potently induces YAP Ser127 dephosphorylation and activates YAP transcriptional activity as assessed by qRT-PCR of YAP direct target gene expression (*CTGF* and *Cyr61*) (Figures 3B and 3C). Taken together, these data indicate that serum-derived S1P and related lipids (dhS1P and LPA) are endogenous lipid compounds that induce YAP dephosphorylation, nuclear localization, and activation. We focused our further studies on S1P as it is the most potent compound identified.

S1P Induces YAP Target Gene Expression

Previously, a panel of ~70 genes were identified as YAP target genes and have been successfully used as molecular signatures to predict YAP activation status (Cordenonsi et al., 2011; Dupont et al., 2011). To probe whether S1P stimulation can activate YAP signature gene expression, we carried out a bioinformatics analysis of published microarray profiling data deposited in the Gene Expression Omnibus (GEO). We found that the YAP signature gene panel is significantly ($p < 0.0001$) activated in mouse liver samples isolated from S1P lyase (SPL)-deficient mice (GSE18745), which have marked accumulation of S1P in plasma and liver (Bektas et al., 2010). The YAP target genes are also significantly ($p < 0.0002$) induced in human embryonic stem cells (ESCs) treated with S1P (GSE7896) (Avery et al., 2008), suggesting that S1P indeed activates YAP transcriptional activity (Figure S11). YAP has been shown to regulate hepatocyte

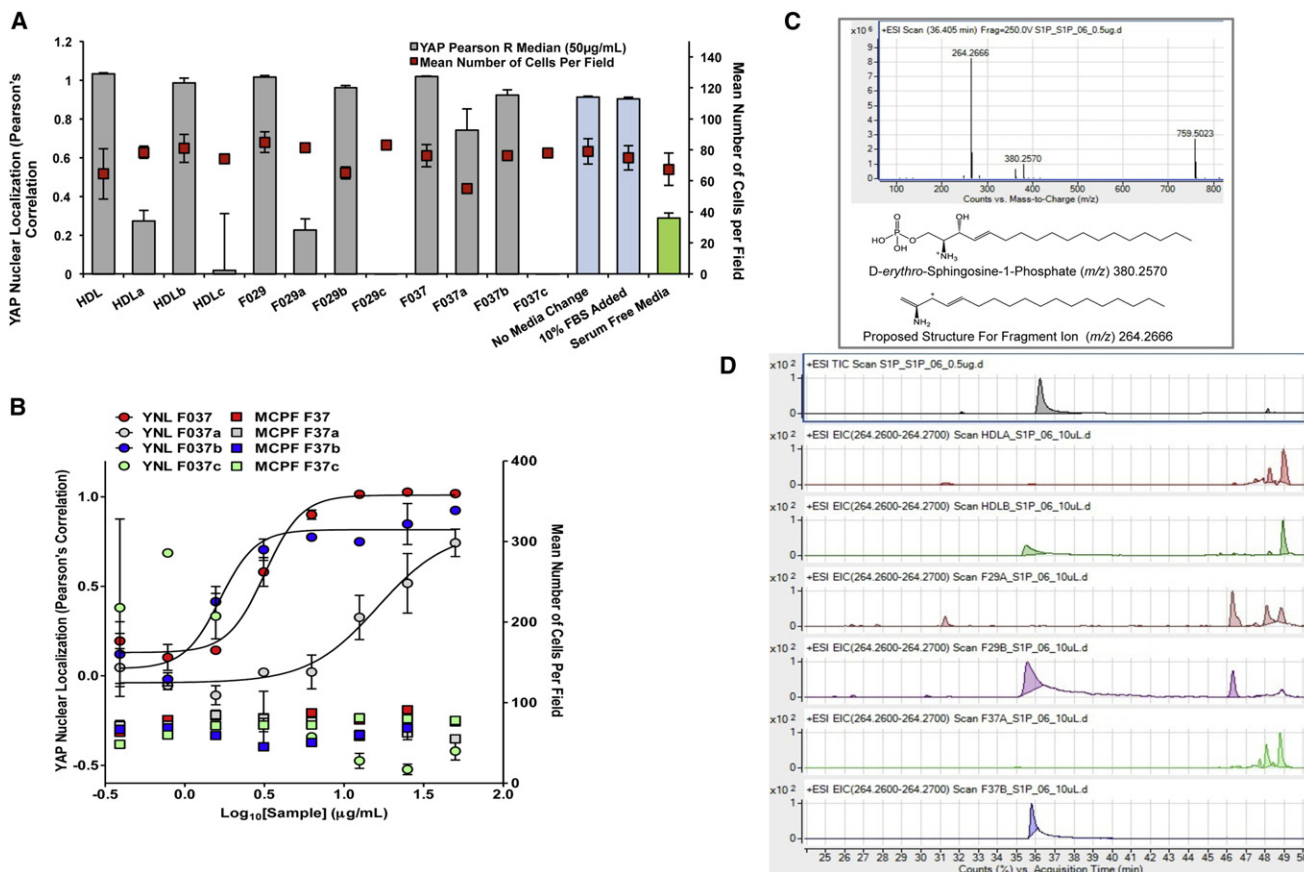


Figure 2. Chemical Isolation and Activity Profiling Lead to the Identification of Serum-Derived Factors that Regulate YAP

(A) Human HDL, Fraction 029, and Fraction 037 (F029 and F037) from FBS purification were further purified by extraction and lipid partitioning methods to give three partitions (partitions a, b, and c). The YAP nuclear localization restoring activity is evaluated in low-density, serum-deprived HEK293A cells at a sample concentration of 50 μg/mL (bar graph). The mean number of cells per field in the imaging analysis for those samples is shown in red squares. (Data are represented as mean ± SEM, n = 3).

(B) Dose-response curves for partitions a (gray), b (blue), and c (green) of Fraction 037 (intact F037, red). Curves with circles represent the YAP nuclear localization (YNL) activity, and the squares represent the mean cell number per field (MCPF) (data are represented as mean ± SEM, n = 3).

(C) Positive mode electrospray ionization (ESI) spectrum of synthetic D-erythro-S1P and proposed structures for the observed ions.

(D) Representative extracted ion chromatograms for the most abundant S1P-derived ion (m/z: 264.26–264.27) for synthetic D-erythro-S1P (top panel, gray) and in descending order: partitions a (HDLa, red) and b (HDLb, dark green) of human HDL; partitions a (F029a, pink) and b (F029b, purple) of Fraction 029 from FBS; and partitions a (F037a, green) and b (F037b, blue) of Fraction 037 from FBS.

See also Figures S5, S6, S7, and S8 and Table S1.

proliferation and embryonic stem cell self-renewal (Lian et al., 2010; Zhou et al., 2009). It would be interesting to further study whether YAP contributes to the effects of S1P in these systems.

S1P Induces YAP Nuclear Localization through S1P₂ Receptor

S1P signals through a family of GPCRs, including S1P_{1–5} (Rosen and Goetzl, 2005). To probe which receptor(s) mediates YAP regulation, we carried out qRT-PCR mRNA expression profiling of the S1P receptors in HEK293A and HaCaT cells, two cell types that respond to S1P stimulation. We found that S1P₂ is the most abundant receptor expressed in both cell lines (Figure S12). S1P₂ antagonist JTE-013 has also been shown to block CTGF and Cyr61 expression, whereas S1P₁ antagonist VPC4416 does not (Kim et al., 2011; Li et al., 2008). Similarly, siRNA-mediated silencing of S1P₂, but not S1P₃, blocks S1P-mediated YAP target

gene expression, suggesting that S1P₂ is the receptor responsible for the effects (Figure S13). S1P₂ is known to couple to the G_{12/13}-family of G proteins, leading to the activation of Rho GTPases, stress fiber formation, and cytoskeletal reorganization (Rosen and Goetzl, 2005). We found that Rho GTPase inhibitor (C3, 3 μg/mL) and ROCK inhibitor (Y-27632, 10 μM) can both block YAP nuclear localization induced by S1P (Figure S14A), suggesting that Rho GTPase-mediated pathway is required for the regulation of YAP. Rho GTPase activation induces F-actin polymerization and stress fiber formation. Consistent with this notion, we found that serum deprivation disrupted F-actin stress fiber in cells, accompanied by YAP cytoplasmic retention (Figures 4A and 4B). S1P (300 nM) restored YAP nuclear localization and F-actin polymerization (Figure 4C). Furthermore, F-actin inhibitor (latrunculin A, 100 nM) can block S1P and LPA-induced YAP nuclear localization (Figures 4D and 4E), suggesting that

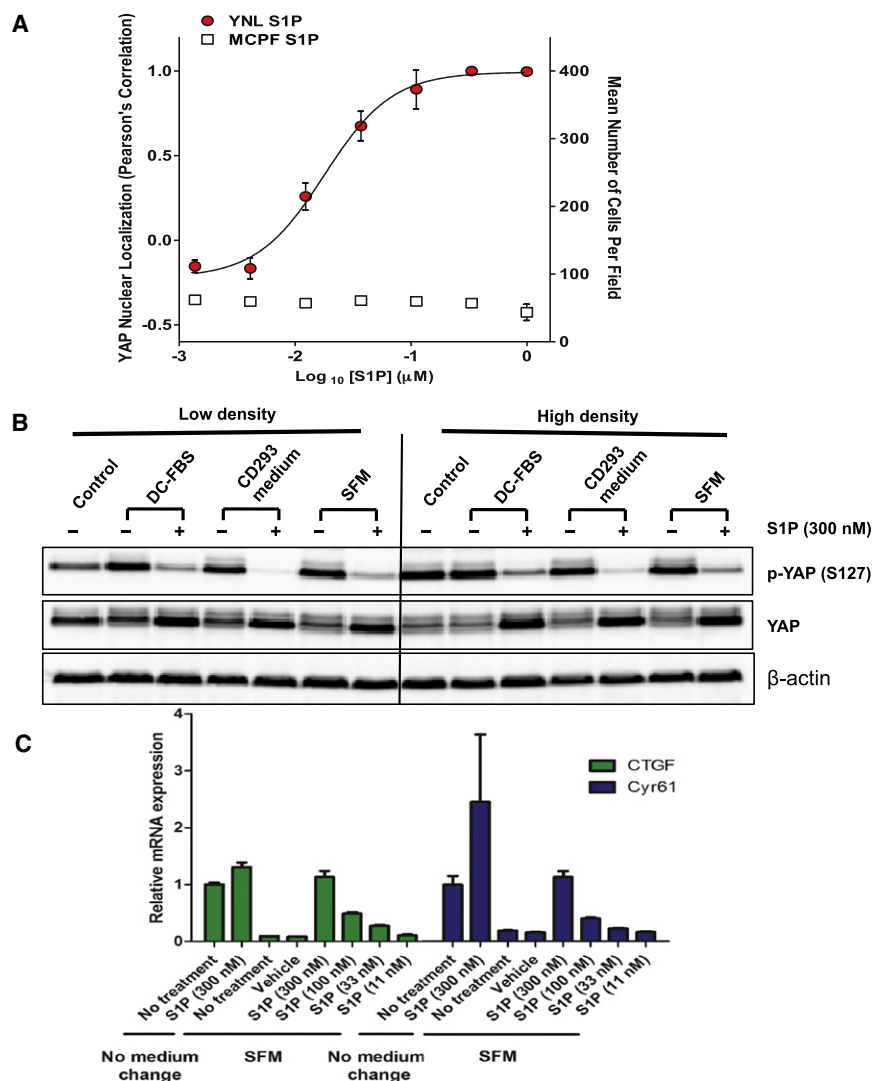


Figure 3. Sphingosine-1-Phosphate Is a Small Molecule Ligand Regulating YAP

(A) Synthetic S1P induces YAP nuclear localization. Curve with circles (red) represents the YAP nuclear localization (YNL) activity, and the open squares represent the mean cell number per field (MCPF). (Data are represented as mean \pm SEM, $n = 3$).

(B) S1P induces YAP Ser127 dephosphorylation independent of cell-cell contact. HEK293A cells were cultured at low density (50% confluency, left) and high density (100% confluency, right). Culture medium was replaced with media containing 10% of charcoal/Dextran-treated FBS (DC-FBS), CD293 synthetic media, or serum-free media (SFM). Cells were treated with 300 nM of S1P (+) or vehicle control (–) for 2 hr.

(C) S1P induces YAP target genes (*CTGF* and *Cyr61*) expression. qRT-PCR analysis of *CTGF* (green) and *Cyr61* (blue) in HEK293A cells with normal culture medium (no medium change), or replenished with serum-free media (SFM) supplemented with vehicle control, or S1P at various doses. (Data are represented as mean \pm SEM, $n = 3$).

See also Figures S9, S10, and S11.

mechanisms of Hippo pathway in multiple species and suggested that deregulation of Hippo pathway is involved in many diseases. Therefore, chemical probes might provide additional tools to study this important signaling in development and diseases and to further explore the therapeutic applications of modulating Hippo signaling. To date, several synthetic chemical compounds have been identified to modulate YAP translocation or YAP-TEAD binding through high-throughput screens; however, their potency and specificity of modulating

S1P and LPA regulate YAP through F-actin polymerization-mediated process. Previously, extracellular matrix (ECM) and mechanical force have also been shown to regulate YAP through Rho GTPase (Dupont et al., 2011). F-actin has also been shown to regulate Hippo-YAP signaling. For example, induction of extra F-actin formation by loss of Capping proteins induces *Drosophila* imaginal discs overgrowth through activation of Yorkie (Fernández et al., 2011; Sansores-Garcia et al., 2011). These reports and our results suggest that F-actin polymerization is a conserved signaling node that integrates signaling inputs, including mechanical forces, cytoskeletal reorganization, and serum/plasma lipids signaling, to regulate YAP activity.

DISCUSSION

Hippo signaling is an emerging pathway involved in tissue homeostasis and tumorigenesis through regulation of YAP, an important factor regulating stem and progenitor cell fate, cell proliferation, survival, and transformation. In the last decade, genetic studies have revealed components and the signaling

YAP need further improvement (Bao et al., 2011; Liu-Chittenden et al., 2012). In this report, we have identified S1P and related serum lipids as chemical factors regulating YAP nuclear localization and activation. To our knowledge, the results establish a new signaling mechanism by which serum lipid factors activate YAP through GPCRs and Rho GTPases. S1P and LPA are known to induce proliferation, migration, and metastasis of certain cancer cells, and their levels are elevated in patients with several types of cancers (Pyne and Pyne, 2010; Xu et al., 1998). The S1P biosynthetic enzyme, sphingosine kinase 1 is also known as an oncogene (Pchejetski et al., 2011; Pyne and Pyne, 2010). Our results suggest that YAP could be a downstream factor mediating such biological activity and that small molecule ligands, such as S1P, LPA, or S1P/LPA receptor modulators, could be useful chemical tools to dissect Hippo signaling.

SIGNIFICANCE

Despite efforts to identify upstream signals that regulate YAP nuclear localization and activity, the endogenous small

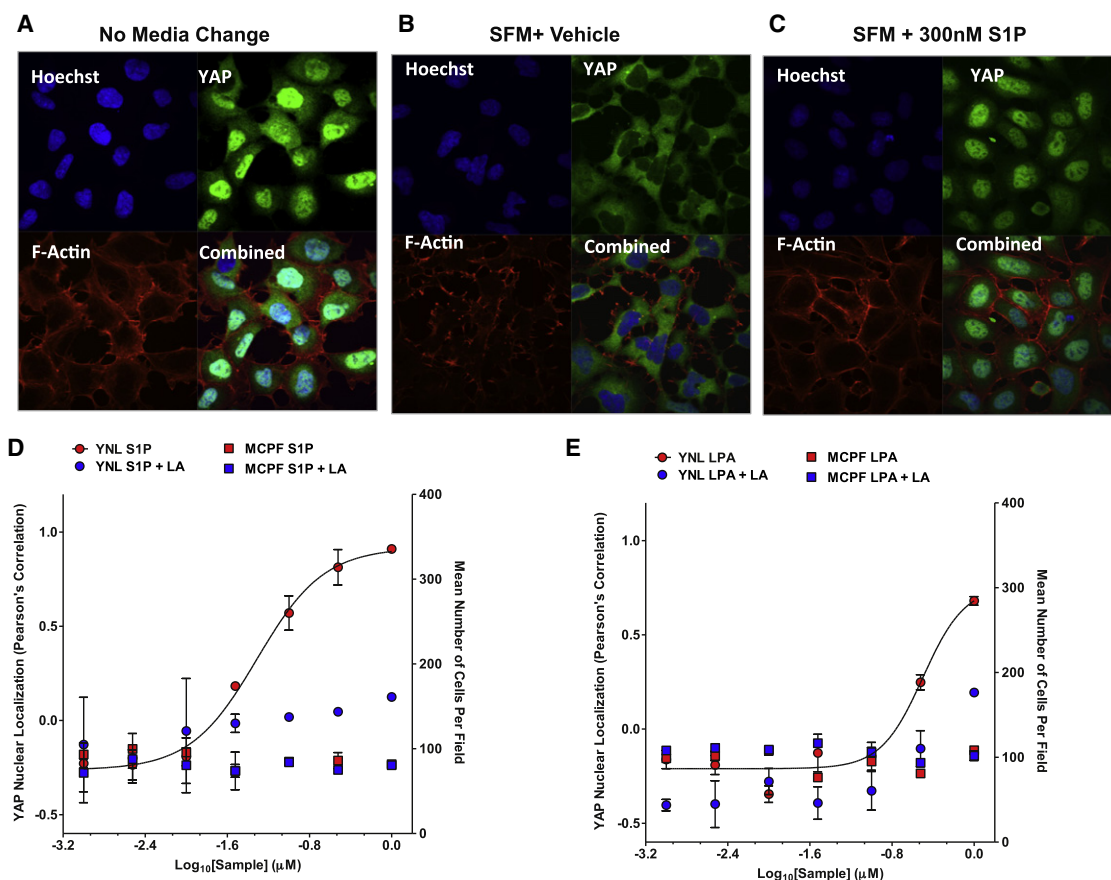


Figure 4. Phospholipids Induce YAP Nuclear Localization through F-actin Polymerization

S1P restores the stress fiber and F-actin polymerization together with YAP nuclear translocation in SFM. HEK293A cells are cultured at low density in normal medium (A), SFM with vehicle control (B), and SFM with S1P (C). Immunofluorescent staining of F-actin stress fibers (red), YAP (green), and nuclei (blue) were shown. F-actin inhibitor Latrunculin A (LA, 100 nM) blocks S1P-induced (D) and LPA-induced (E) YAP nuclear localization ($n = 3$, mean \pm SEM). See also Figures S12, S13, and S14.

molecules that regulate YAP are still elusive in mammals. Here, we report the discovery of serum lipids as potent activators of YAP, providing insights to the regulation of Hippo signaling and chemical tools to perturb YAP activity. We also demonstrated that chemical isolation, activity-guided profiling, and high-throughput screen are powerful tools to identify biologically active endogenous ligands that regulate cell signaling. Further studies on the S1P and LPA-regulated YAP activation might reveal novel physiological and pathological roles of these bioactive lipids.

EXPERIMENTAL PROCEDURES

Activity Profiling of Serum Fractions and Synthetic Lipids

For all experiments, cells were cultured and plated in Dulbecco's modified Eagle's medium (DMEM) + 10% FBS in 384-well plates and allowed to attach overnight. Plating density was either 5×10^4 cells/ml for low density (~50% confluency) or 2×10^5 cells/ml for high density (~100% confluency). Attached cells were washed five times with serum-free DMEM (SFM) at volumes equivalent to the initial plating volume. Serum, serum-derived fractions, or purified lipids were then added back and incubated for 2 hr. Untreated serum was diluted in DMEM; lyophilized fractions were dissolved in DMEM + 3% DMSO; and lipid extracts were first dissolved in methanol and dried under

N_2 (g) to create a thin film, dissolved in 4 mg/ml fatty acid-free (FAF) BSA, and diluted into DMEM. All purified lipids were initially dissolved in solvents in accordance with the manufacturer's instructions, dried to make thin films, dissolved in 4 mg/ml FAF BSA, and diluted in DMEM.

Immunofluorescence Staining

Cells were fixed in 4% paraformaldehyde at room temperature for 15 min, prior to permeabilization with 0.1% Triton X-100. Blocking and primary antibody (anti-YAP, mouse monoclonal antibody [M01] from Abgent, San Diego, CA, USA) incubation was combined in one step by adding antibody prepared in 5% bovine serum albumin (BSA) solution (Sigma-Aldrich). After 2 hr incubation, cells were then washed with PBS and incubated in Alexa Fluor 488 donkey anti-mouse IgG and Hoechst 33342 nucleus dye (Invitrogen, Carlsbad, CA, USA) for 1 hr, followed by image acquisition by high-throughput confocal microscopy (Opera High Content Screening System, Waltham, MA, USA). Nine images/well were captured using a $10 \times$ PlanApo/0.3 NA objective at a resolution of $\sim 0.65 \mu\text{m}/\text{pixels}$.

Additional methods are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes fourteen figures, one table, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.chembiol.2012.07.005>.

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