Thromboxane A2 Activates YAP/TAZ Protein to Induce Vascular Smooth Muscle Cell Proliferation and Migration

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The thromboxane A2 receptor (TP) has been implicated in restenosis after vascular injury, which induces vascular smooth muscle cell (VSMC) migration and proliferation. However, the mechanism for this process is largely unknown. In this study, we report that TP signaling induces VSMC migration and proliferation through activating YAP/TAZ, two major downstream effectors of the Hippo signaling pathway. The TP-specific agonists [1S-[1E,3S],4 a]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (I-BOP) and 9,11-dideoxy-9α,11α-methanoepoxyprosta-5Z,13E-dien-1-oic acid (U-46619) induce YAP/TAZ activation in multiple cell lines, including VSMCs. YAP/TAZ activation induced by I-BOP or knockdown of the downstream G proteins Gα12/13,3,11, and other trimeric G proteins regulates downstream effectors (11). TP is expressed as two isoforms in humans: TPα and TPβ. Besides TxA2, prostaglandin H2, isoprostanes (such as 8-iso-prostaglandin F2α), and hydroxyeicosatetraenoic acids can also activate TP receptors (12–14).

In addition to platelet activation, TxA2 or TP receptor is also known to promote cell migration and proliferation of vascular smooth muscle cells (VSMCs) (15–20), an important process that is involved in a number of vascular diseases, such as post-angioplasty restenosis and atherosclerosis (21). The proliferative response of VSMCs to vascular injury is markedly exaggerated in transgenic mice with vascular overexpression of the human TPα receptor, which can be inhibited by the TP-specific antagonist S18886 (6). Moreover, injury-induced vascular proliferation and platelet activation are suppressed in mice genetically deficient in TP receptor (6). In house models of atherosclerosis, both pharmacological antagonism and TP receptor deletion delay lesion development (22–24). Taken together, these previous studies demonstrate that TxA2 and TP receptor contribute to VSMCs mediating vascular disease, although the molecular mechanism is largely unknown.

The abbreviations used are: TxA2, thromboxane A2; TP, thromboxane A2 receptor; GPCR, G protein-coupled receptor; VSMC, vascular smooth muscle cell; CTGF, connective tissue growth factor; MAVSVMC, mouse aortic vascular smooth muscle cell; dKO, double knockout; U-46619, 9,11-dideoxy-9α,11α-methanoepoxyprosta-5Z,13E-dien-1-oic acid; SQ-29548, [1S-[1E,3S],4 a]-7-[3-[2-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; I-BOP, [1S-[1E,3S],4 a]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; EdU, 5-ethynyl-2′-deoxyuridine; LATS, large tumor suppressor kinase; MAP4K, mitogen-activated protein kinase kinase kinase kinase; TEAD, TEA domain transcription factor; CRISPR, clustered regularly interspaced short palindromic repeats.
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The Hippo signaling pathway plays a key role in the regulation of organ size and tissue homeostasis (25). Core components of the mammalian Hippo pathway include MST1/2 and their adaptor protein SAV1, LATS1/2 and their adaptor proteins MOB1A/1B, and two downstream transcriptional effectors, YAP/TAZ (25). MST1/2 phosphorylate and activate LATS1/2 kinases, which in turn phosphorylate and inhibit YAP/TAZ. Recently, MAP4Ks have been shown to be core components of the Hippo pathway, and they function in parallel to MST1/2 to phosphorylate and activate LATS1/2 (26, 27). Phosphorylation of YAP at Ser-127 results in cytoplasmic sequestration because of 14-3-3 binding (28, 29). The dephosphorylated YAP/TAZ translocate into the nucleus and interact with the TEAD family transcription factors to induce target genes, such as connective tissue growth factor (CTGF) and cysteine-rich angiogenic inducer 61 (CYR61), thereby promoting cell proliferation, migration, and survival (30–32). Deregulation of the Hippo pathway has been observed in various human cancers and is often correlated with a poor prognosis (33). Upstream signals of the Hippo pathway had been elusive until recent studies established that GPCRs relay extracellular signals to the Hippo pathway (34–38). Ligands signaling through GPCRs coupled to Gα12/13, Gαq/11, or Gαi, activate YAP/TAZ, whereas ligands signaling through Gαs-coupled GPCRs suppress YAP/TAZ activity (36). Therefore, stimulation of different GPCRs can result in either activation or inhibition of YAP/TAZ.

Interestingly, emerging evidence shows that YAP is induced after arterial injury and that its activation promotes smooth muscle phenotypic switching and neointima formation (39, 40). This led us to investigate the function of YAP/TAZ in TxA2 and TP receptor-induced cellular signaling and VSMC migration and proliferation. In this study, we show that TP activation increases YAP/TAZ activity in VSMCs and other cell types via Gα12/13. Importantly, YAP/TAZ are essential for TP-induced VSMC proliferation and migration, providing a plausible mechanism for VSMC-mediated vascular diseases.

Results

Stimulation of TP Induces YAP/TAZ Dephosphorylation and Nuclear Accumulation—To study TP regulation on YAP/TAZ activity, we treated cells with the TP agonist I-BOP because the physiological TP ligand TxA2 is extremely unstable. In serum-starved HeLa cells that express TP, YAP was highly phosphorylated. Addition of I-BOP resulted in a significant decrease in YAP phosphorylation, as determined by immunoblotting with a phospho-YAP antibody (Ser-127) (Fig. 1, A and B). I-BOP also induced TAZ dephosphorylation, as indicated by the differential migration on phos-tag gels (Fig. 1B). Moreover, I-BOP-induced YAP/TAZ dephosphorylation was rapid and transient, as the YAP/TAZ dephosphorylation was visible 15 min after stimulation and partially recovered 4 h after I-BOP treatment (Fig. 1B). In addition, U-46619, another TP-specific agonist, could also induce YAP/TAZ dephosphorylation in a dose- and time-dependent manner (supplemental Fig. 1, A and B).

Phosphorylation of YAP at Ser-127 by LATS1/2 leads to binding with 14-3-3 and cytoplasmic sequestration of YAP, and dephosphorylated YAP accumulates in the nucleus and induces gene expression by interacting with the transcription factors TEAD1–4 (29, 31). Similarly, phosphorylation of TAZ at Ser-89 by LATS1/2 also induces cytoplasmic localization (30). As expected, I-BOP treatment caused significant nuclear accumulation of YAP and TAZ (Fig. 1, C and D; supplemental Fig. 1, C). Phosphorylation of YAP/TAZ was dose-dependent, and YAP/TAZ dephosphorylation was evident when as little as 0.1 nmol/liter I-BOP was added to primary MAVSMCs (Fig. 1H). Similarly, the expression of YAP/TAZ target genes, such as CTGF, CYR61, TAGLN, and EDN1, was significantly induced by I-BOP or U-46619 treatment (supplemental Fig. 1, D and E). Accordingly, the CYR61 protein level was also increased upon I-BOP stimulation (Fig. 1B).

The effect of TP activation on YAP/TAZ was consistently observed in multiple cell lines, including MDA-MB-231, SW480, and HEK293A (supplemental Fig. 1, F–I). Notably, I-BOP also induces YAP/TAZ dephosphorylation in VSMCs, such as the T/G HA-VSMC cell line and primary mouse aortic VSMCs (MAVSMC) (Fig. 1, F–H). The effect of I-BOP on YAP/TAZ phosphorylation was dose-dependent, and YAP/TAZ dephosphorylation was evident when as little as 0.1 nmol/liter I-BOP was added to primary MAVSMCs (Fig. 1H). Similarly, the expression of YAP/TAZ target genes, such as CTGF, CYR61, TAGLN, and EDN1, was significantly induced by I-BOP in T/G HA-VSMCs (Fig. 1I). Based on the above data, we conclude that stimulation of TP activates YAP/TAZ by inducing their dephosphorylation and nuclear translocation.

I-BOP Acts through TP and Gα12/13 to Activate YAP/TAZ—Because of the unstable property of TxA2, I-BOP and U-46619 were used to treat cells. To exclude that these chemicals have an unexpected effect on YAP/TAZ independent of TP, we pretreated cells with the TP-specific antagonist SQ-29548 followed by I-BOP treatment. As shown in Fig. 2A, I-BOP-induced YAP/TAZ dephosphorylation was blocked by SQ-29548 in T/G HA-VSMCs. HEK293A cells are not very sensitive to I-BOP stimulation, likely because of the low level of TP expression. Ectopic expression of TPα receptor rendered YAP/TAZ more sensitive to 1 nmol/liter I-BOP treatment, a concentration that had a minor effect on YAP/TAZ phosphorylation in the control HEK293A cells (Fig. 2B). A similar phenomenon was observed when TPβ was overexpressed in U2OS cells (supplemental Fig. 2). These data indicate that I-BOP acts through both isoforms of TP receptor to activate YAP/TAZ.

To further confirm the role of endogenous TP in YAP/TAZ regulation, we generated TP KO cells using the CRISPR/Cas9 genome editing system. Two independent TP KO cell lines were generated, and the TP deletion was verified by Sanger sequencing (supplemental Fig. 3). TP knockout completely blocked I-BOP-induced YAP/TAZ dephosphorylation and YAP nuclear accumulation (Fig. 2, C and D). Consistently, I-BOP was unable to induce the expression of YAP target genes in TP KO cells (Fig. 2E). These data show that I-BOP stimulates TP receptor to induce YAP/TAZ activation.

TP receptor activates several trimeric Gα proteins, including Gαq/11 and Gα12/13, to initiate intracellular signaling pathways (11). To determine which Gα proteins are involved in YAP/TAZ regulation, Gαq/11 or Gα12/13 were knocked down by
RNAi in HEK293A cells (Fig. 2F). Knockdown of Gα_{12/13} strongly blocked YAP/TAZ dephosphorylation in response to I-BOP, whereas knockdown of Gα_{q/11} had little effect on I-BOP-induced YAP/TAZ dephosphorylation (Fig. 2F). Consistently, I-BOP induced YAP nuclear accumulation in control siRNA- and Gα_{12/13} siRNA-transfected cells but not in Gα_{q/11}
FIGURE 2. TP agonist activates YAP/TAZ via TP receptor and $\alpha_{12/13}$. A, the TP antagonist SQ-29548 blocks YAP/TAZ dephosphorylation induced by I-BOP. Serum-starved T/G HA-VSMCs were pretreated with the TP-specific antagonist SQ-29548 (1 μmol/liter) for 3 h and then stimulated with I-BOP (1 nmol/liter) for 1 h. Cell lysates were subjected to immunoblotting with the indicated antibodies. B, ectopic expression of TP receptor renders HEK293A cells sensitive to I-BOP treatment. HEK293A cells were transiently transfected with the indicated plasmids and stimulated with different concentrations of I-BOP for 1 h. Immunoblotting was performed with the indicated antibodies. C, knockout of TP blocks I-BOP-induced YAP/TAZ dephosphorylation. Wild-type or TP KO HEK293A cells, which were verified by genomic DNA sequencing (supplemental Fig. 3), were seeded with a density of $8 \times 10^4$ cells/cm$^2$ for 24 h and then treated with 10 nmol/liter I-BOP for 1 h. Immunoblotting was performed with the indicated antibodies. Con, control. D, TP KO abolishes I-BOP-induced YAP nuclear translocation. Stimulation conditions were the same as in C. YAP localization was determined by immunofluorescence. Scale bars = 30 μm. E, TP KO blocks I-BOP-induced target gene expression. Wild-type or TP KO HEK293A cells were treated with 10 nmol/liter I-BOP for 2 h. mRNA levels of CTGF and CYR61 were measured by quantitative PCR. F, I-BOP activates YAP/TAZ via $\alpha_{12/13}$. HEK293A cells were transfected with the indicated siRNAs. A mixture of two independent oligonucleotides was used for one gene. Two days after transfection, cells were treated with 10 nmol/liter I-BOP for 1 h. Cells were lysed and subjected to immunoblotting with the indicated antibodies. G, knockdown of $\alpha_{12/13}$ blocks I-BOP-induced YAP nuclear localization. Stimulation conditions were similar as in F. Immunofluorescence staining for endogenous YAP (green), F-actin (red), and DNA (blue) is presented. Scale bars = 20 μm.
siRNA-transfected cells (Fig. 2G, supplemental Fig. 4). Taken together, we conclude that TP signals through Gα12/13 to induce YAP/TAZ dephosphorylation and activation.

I-BOP Modulates YAP/TAZ Dephosphorylation via Rho GTPase and the Actin Cytoskeleton—Rho GTPase is a known downstream signaling module of Gα12/13, which directly interacts and activates the Rho guanine nucleotide exchange factor. We therefore tested the role of Rho GTPase in I-BOP-induced YAP/TAZ dephosphorylation. Expression of Rho GDP dissociation inhibitor, which binds to Rho GTPase and inhibits GTPase cycling, suppressed I-BOP-induced YAP/TAZ dephosphorylation (Fig. 3A). Likewise, botulinum toxin C3, a specific inhibitor of Rho GTPase, strongly blocked YAP/TAZ dephosphorylation in response to I-BOP or U-46619 treatment (Fig. 3B; supplemental Fig. 5, A and B). Consistently, C3 treatment blocked I-BOP- or U-46619-induced YAP nuclear translocation (Fig. 3C, supplemental Fig. 5C). These data indicate that Rho GTPase is required for TP to activate YAP/TAZ.

The major function of Rho GTPase is to modulate the actin cytoskeleton, particularly stress fiber formation. Recently studies have shown that the actin cytoskeleton plays an important role in the Hippo pathway (41–45). We therefore tested whether cytoskeletal reorganization contributes to YAP/TAZ activation by TP agonists. Latrunculin B, an F-actin-disrupting reagent, blocked I-BOP- or U-46619-induced YAP/TAZ dephosphorylation (Fig. 3D, supplemental Fig. 5D). Consistent with changes in YAP phosphorylation, Latrunculin B treatment also prevented YAP nuclear accumulation in response to I-BOP or U-46619 (Fig. 3E, supplemental Fig. 5E). Moreover, I-BOP or U-46619 induced actin stress fiber and YAP nuclear translocation (Figs. 2G and 3, C and E; supplemental Fig. 5, C and E). Knockdown of Gα12/13 or treatment with C3 or Latrunculin B disrupted F-actin formation and YAP nuclear accumulation (Figs. 2G and 3, C and E; supplemental Fig. 5, C and E). These results indicate that TP acts through Rho GTPase and the actin cytoskeleton to affect YAP/TAZ phosphorylation.

I-BOP Inhibits LATS—LATS1/2 are the kinases directly responsible for YAP/TAZ phosphorylation. The phosphorylation of the activation loop (Ser-909/Ser-872 for LATS1/2) correlates with changes in YAP phosphorylation and is a known substrate for LATS1/2 kinases. We therefore measured LATS1/2 kinase activity in vitro to determine whether TP inhibits LATS1/2 activity. As expected, I-BOP markedly inhibited LATS1/2 kinase activity in vitro in a dose-dependent manner (Fig. 4A). Consistent with these results, we observed that I-BOP treatment significantly decreased LATS1/2 kinase activity in cells (Fig. 4B). These data indicate that TP inhibits LATS1/2 activity in vivo, which is consistent with the findings from in vitro experiments.

I-BOP Inhibits YAP/TAZ Phosphorylation—The inhibition of LATS1/2 by TP suggests that TP may target YAP/TAZ phosphorylation. To test this hypothesis, we measured YAP/TAZ phosphorylation in cells treated with I-BOP. As expected, I-BOP treatment significantly decreased YAP/TAZ phosphorylation (Fig. 4C). These results indicate that TP inhibits YAP/TAZ phosphorylation in vivo.

These findings provide a comprehensive understanding of the molecular mechanisms by which TP regulates YAP/TAZ phosphorylation and nuclear localization. TP activates YAP/TAZ phosphorylation and nuclear localization through a complex signaling pathway involving Rho GTPase and the actin cytoskeleton. This pathway is modulated by a variety of factors, including Gα12/13, Rho GTPase, and the actin cytoskeleton. These results have important implications for understanding the role of TP in regulating YAP/TAZ phosphorylation and nuclear localization in vivo.
I-BOP could not affect YAP/TAZ phosphorylation in LATS1/2-dKO cells (supplemental Fig. 6), suggesting that LATS1/2 are required for I-BOP-induced YAP/TAZ dephosphorylation. MST1/2 and MAP4Ks are responsible for LATS kinase activation in response to upstream signals (26, 27, 46). To test whether MST1/2 or MAP4Ks are involved in I-BOP-induced YAP/TAZ dephosphorylation, we used MST1/2 double knockout (MST1/2 dKO) and combined deletion of MST1/2 and MAP4Ks in I-BOP-induced YAP/TAZ dephosphorylation. Wild-type or MM-9KO HEK293A cells were transfected with the indicated plasmids and treated with I-BOP (10 nmol/liter) for 1 h. The cell lysates were subjected to immunoprecipitation with FLAG beads. The immunoprecipitated FLAG-LATS1 was measured for in vitro kinase assays using GST-YAP as a substrate. The phosphorylation of LATS1 and GST-YAP was detected by immunoblotting with the indicated antibodies.

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YAP/TAZ Are Required for TP to Stimulate VSMC DNA Synthesis and Cell Migration—TP has been shown to promote neointima formation, which is caused by VSMC migration and proliferation (6, 9, 47). Because YAP/TAZ activity is significantly activated upon TP stimulation in VSMCs, we investigated whether YAP/TAZ activation is involved in the proliferation and migration of VSMCs. YAP/TAZ were knocked down in T/G HA-VSMCs by inducible shRNA and siRNA, respectively. The knockdown efficiency was confirmed by immunoblotting of protein levels (Fig. 5A). Knockdown of YAP/TAZ significantly suppressed the mRNA induction of CTGF and CYR61 in response to I-BOP (Fig. 5B). I-BOP strongly induced cell migration in control cells, whereas this effect was significantly suppressed in YAP/TAZ double knockdown cells (Fig. 5, C and D). In addition, I-BOP-induced VSMC DNA synthesis was also suppressed by YAP/TAZ double knockdown, as deter-
FIGURE 5. YAP/TAZ mediate the effect of TP in gene induction, DNA synthesis, and cell migration in VSMCs. A, knockdown of YAP/TAZ by shRNAs and siRNAs in T/G HA-VSMCs. Cells were transfected with the indicated siRNAs. 48 h after transfection and doxycycline (Dox) induction, cells were lysed and subjected to immunoblotting with the indicated antibodies. B, YAP/TAZ are required for I-BOP-induced gene expression. T/G HA-VSMCs were transfected with the indicated siRNAs and serum-starved for 24h in the presence of doxycycline. After treatment with I-BOP (1 nmol/liter) for 1 h, mRNA levels of CTGF and CYR61 were measured by real-time PCR. The numbers next to each treatment conditions will be used to label C–E. C, YAP/TAZ are required for I-BOP-induced cell migration. The treatment conditions for each panel are the same as in B. After stimulation with I-BOP (1 nmol/liter) for 4 h, cell migration was performed by transwell cell migration assay. Representative images are shown. Con, control. D, quantification result of the data in C. * , p < 0.05. Statistical analysis is described under “Experimental Procedures.” E, YAP/TAZ are required for I-BOP-induced DNA synthesis. T/G HA-VSMCs were transfected with the indicated siRNAs and serum-starved for 20 h. After stimulation with I-BOP (1 nmol/liter) for 4 h, cell migration was determined by transwell cell migration assay. Representative images are shown. H, quantification result of the data in G. * , p < 0.05.
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FIGURE 6. A proposed model for thromboxane A2 receptor in the regulation of YAP/TAZ activities.

mined by EdU incorporation (Fig. 5E, supplemental Fig. 8A). To further support the important role of the TP-YAP/TAZ axis in VSMCs, we isolated and analyzed primary MAVSMCs. Similar Yap/Taz knockdown experiments were performed in primary MAVSMCs. Consistently, knockdown of Yap/Taz in primary MAVSMCs also inhibited cell migration induced by I-BOP (Fig. 5, F–H). In addition, we observed that YAP/TAZ knockdown suppressed I-BOP-induced gene expression and cell migration in HeLa cells (supplemental Fig. 8, B–D). Taken together, these results demonstrate that YAP/TAZ play an important role in TP-mediated gene induction, cell proliferation, and migration in VSMCs.

Discussion

TxA2 is involved in multiple physiological and pathophysiological processes, including thrombosis, asthma, myocardial infarction, inflammation, atherosclerosis, and the response to vascular injury (11). TxA2 exerts its biological activity via its cognate TP receptor. In this study, we demonstrate that the Hippo pathway is a crucial downstream signaling module of TP receptor, a classical GPCR. TP agonists significantly activate YAP/TAZ in multiple cells lines, including VSMCs. Our data also demonstrate that activation of TP couples to Goα12/13 to trigger the activation of Rho GTPase, which modulates the actin cytoskeleton to inhibit LATS1/2 kinase activity, resulting in YAP/TAZ dephosphorylation and activation (Fig. 6). In this signaling cascade, both MST1/2 and MAP4Ks, the major kinases for LATS1/2, are involved in Hippo pathway regulation by TP. Our studies indicate a functional role of the Hippo pathway and YAP/TAZ in mediating the physiological and pathological functions of thromboxane and its receptor TP.

In addition to TxA2, there are four other major prostaglandins generated from arachidonic acid in vivo, including prostaglandin D2, prostaglandin E2, prostaglandin F2α, and prostacyclin (48). They all exert their effects via corresponding GPCRs. It is interesting to speculate that YAP/TAZ may play a similar role in physiological and disease processes that are regulated by prostaglandins, such as inflammation, atherosclerosis, and cancer (49). For instance, the prostacyclin receptor couples to Goα and stimulates cAMP production and PKA activation (50). Previously we reported that cAMP acts through PKA to stimulate LATS kinases and inhibit YAP (51). Given that TxA2 and prostacyclin are antagonistic in their actions in cardiovascular disease (6), it will be interesting to determine whether prostacyclin inhibits YAP/TAZ, thereby antagonizing the effect of TxA2, which activates YAP/TAZ, as shown in this report, in cardiovascular disease.

Isoprostanes are prostaglandin-like compounds formed from the free radical-catalyzed peroxidation of unsaturated fatty acids, such as arachidonic acid, that are recognized not only as reliable markers of oxidative stress but also as important mediators of various diseases (52). Increased formation of isoprostanes has been observed in diseases that are linked to oxidative stress, such as cardiovascular disease and cancer (52). As mentioned above, isoprostanes act, at least partially, via TP to exert their physiological effects. So one may speculate that YAP/TAZ also play a role in isoprostane-mediated pathophysiological effects. Indeed, we observed that 8-iso-prostaglandin F2α, a type of isoprostanes, could induce YAP/TAZ dephosphorylation in VSMCs. Future studies are needed to delineate the involvement and potential role of YAP/TAZ in isoprostane-induced physiological processes.

Our study shows that YAP/TAZ are required for TP-stimulated VSMC DNA synthesis and cell migration. The connection between TP receptor and the Hippo pathway in vascular smooth muscle cells has important physiological implications. During vascular injury, activated platelets or other cells produce TxA2, which in turn promotes platelet activation and aggregation (4, 53). Besides TxA2, isoprostanes may also act through the TP receptor on the surface of smooth muscle cells and initiate the migration from media to intima via YAP/TAZ activation. Previous studies have reported that YAP is activated in VSMCs of the neointima (39). We propose that thromboxane acts through TP to induce YAP/TAZ activation to promote normal physiological wound healing in response to vascular injury. However, too much YAP/TAZ activation by TP under pathological conditions may lead to VSMC overgrowth, thereby contributing to neointima formation and restenosis. Notably, CTGF and CYR61 are strongly induced upon TP stimulation (Figs. 2E and 5B). Both CTGF and CYR61 have been shown to promote atherosclerotic lesion development and neointimal hyperplasia (54–55).
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Experimental Procedures

Plasmids—The plasmids pCMV-HA-YAP2, FLAG-LATS1, FLAG-MST2, and FLAG-MAP4K2 and GFP-GDP dissociation inhibitor were described before (26, 51, 59). TPα/β were amplified from cDNA and then subcloned into pRK7-N-FLAG and pQCXIH vectors with the restriction enzymes BamHI/EcoRI.

Antibodies—Antibodies for YAP (610922) was purchased from BD Transduction Laboratories. TAZ (HPA007415) and Vinculin (V9264) antibodies were purchased from NeoMarkers, and anti-FLAG antibodies were from Cell Signaling Technology. The LATS1 (A300-477A) and YAP (A302-308A) antibodies used for immunoprecipitation and LATS2 (A300-479A) antibody were from Bethyl Laboratories. TAZ (HPA007415) and Vinculin (V9264) antibodies were obtained from Sigma-Aldrich. Anti-TEAD1 (610922) was purchased from BD Transduction Laboratories. Antibodies for Goq11 (Cys-19, sc-392) and Go12 (Ser-20, sc-409) and the HA-probe antibody (sc-7392) were from Santa Cruz Biotechnology. The tubulin antibody (581P) was purchased from sc-409) and the HA-probe antibody (sc-7392) were from Santa Cruz Biotechnology. The tubulin antibody (581P) was purchased from Sigma-Aldrich. Anti-β-actin (A00702), Lamin A/C (A01455), and anti-FLAG antibodies were purchased from GeneScript. The GFP tag (7C9, M20004) was from Abmart. The anti-G protein α13 antibody (EPR5436, ab128900) was obtained from Abcam.

Chemicals—U-46619 (9,11-dideoxy-9α,11α-methanoepoxyprosta-5Z,13E-dien-1-oic acid) and SQ-29548 ([1S-[1α,2α(Z),3α,4α])-7-[[2-[(phenylamino)carbonyl]hydrazino]-methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-hepentoic acid) were purchased from Sigma-Aldrich. Anti-TEAD1 (610922) was purchased from BD Transduction Laboratories. Antibodies for Goq11 (Cys-19, sc-392) and Go12 (Ser-20, sc-409) and the HA-probe antibody (sc-7392) were from Santa Cruz Biotechnology. The tubulin antibody (581P) was purchased from NeoMarkers, and anti-β-actin (A00702), Lamin A/C (A01455), and anti-FLAG antibodies were purchased from GeneScript. The GFP tag (7C9, M20004) was from Abmart. The anti-G protein α13 antibody (EPR5436, ab128900) was obtained from Abcam.

Transfection and Lentiviral and Retroviral Infection—Cells were transfected with plasmid DNA using PEI. To generate U2OS cells stably expressing TPα/β, retroviruses carrying pQCXIH empty vector or pQCXIH-TPα/β were produced in HEK293T cells using vesicular stomatitis virus G and GAG as packaging plasmids. The virus supernatant was filtered through a 0.45-μm filter and used to infect targeting cells in the presence of 8 μg/ml Polybrene. Stable cell pools were selected with 50 μg/ml hygromycin B (Amresco) for 5 days. For tetracycline-inducible shRNA expression, a lentivirus containing shRNAs in the pTRIPZ vector was made in HEK293T cells using pMD2.g and psPAX2 as packaging plasmids. Viral infection was performed as described above except for selection with 1 μg/ml puromycin (Amresco) for 5 days. Expression of shRNA was induced by adding 1 μg/ml doxycycline for 48 h. The shRNA sequences against YAP were as follows: YAP#1, TTCTTTATCT-AAGCTTGTGGC; YAP#2, TGGTCAAGATACCTTCTAA.

RNA Interference—siRNAs targeting GNA11, GNA12, GNA13, YAP1/YAPI, and WWTR1/Wwtr1 were from GenePharma and were delivered into cells using RNAiMAX (Invitrogen) according to the instructions of the manufacturer. The sequences of all siRNAs used in this study were as follows: siGNA11#1, GACACCGAGAATATCCGCTT; siGNA11#2, CTAT-GATAGACGACGAGA; siGNA11#1, GTCGAAGATC-CTCTAAGATG; siGNA11#2, GCTCACCTCAAGGAT-TCAA; siGNA12#1, GCAGCAACCACCTTCTGCAA; siGNA12#2, GGATGTCTCAGTAGGCTT; siGNA13#1, GTCGAAGG-AAGCCTCTA; siGNA13#2, CCTGCTATAAGGACATT; siYAPI#1, CCCAGTTAAATGTTCACCAAT; siYAPI#2, CAGGATATCTACCAACCAA; siTAZ#1, CACGCAATCTCAGGATGAAT; siTAZ#2, CAGCTGAGATACCTTCTGAAT; siYap1#1, GAAGCGCTGAGTTCCGAAATC; siYap1#2, TGGACACTGACAGTATGAT; and siTaz2, CACTGAGCAGAGATGAGAT.

RNA Extraction and Real-time PCR—Cells were washed with cold PBS, and total RNA was isolated using TRlzol reagent following the instructions of the manufacturer (Invitrogen). 1 μg of RNA was reverse-transcribed to cDNA with oligo(dT) prim-
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ers (Transgene). cDNA was then diluted and subjected to real-time PCR with gene-specific primers using SYBR Premix Ex Taq (TaKaRa) and the 7500 real-time PCR system (Applied Biosystems). The primer pairs used in this study were as follows: Actin, GCGCAGAGATGCAGAAGGATCA/AA- GCATTGGTGAGCGATGGA; CTGF, CCAATGACAGGC- GCCCTCTG/TCGTTGAGCAGAAAGCTC; CYR61, AGCC- GCATTTGCGGTGGACGATGGA; CTGF, CCAATGACAGGC- GCCCTCTG/TCGTTGAGCAGAAAGCTC; TP

Immunoprecipitation—Cells were lysed using lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1.5 mM Na3VO4, 1 mM PMSF, and protease inhibitors). Cell lysates were centrifuged at 12,000 × g for 15 min at 4 °C. The supernatants were incubated with YAP antibody (Bethyl Laboratories) for 2 h, followed by protein A-agarose bead incubation for 1 h. Immunoprecipitates were washed three times with lysis buffer, and proteins were boiled with SDS-PAGE sample buffer.

Immunoblotting—Cells were lysed in SDS sample buffer and denatured by heating on 99 °C for 10 min. Immunoblotting was performed in 8% or 10% BisTris polyacrylamide gel according to the standard protocol. The phos-tag reagents were purchased from Wako Chemicals, and gels containing phos-tag were prepared following the instructions of the manufacturer. YAP and TAZ proteins can be separated into multiple bands in phos-tag gels depending on differential phosphorylation levels, with phosphorylated YAP/TAZ migrating more slowly. Scoring and quantifying.

EdU Incorporation Assay—T/G HA-VSMCs grown on 24-well plates were serum-starved for 24 h. I-BOP (1 nmol/liter) was added every 8 h for 3 times. 10 μmol/liter EdU (5-ethynyl-2'-deoxyuridine) was added to the culture medium for 4 h. After labeling, cells were fixed with 4% paraformaldehyde for 20 min, followed by permeabilization with 0.5% Triton X-100 in PBS for 20 min at room temperature. Cells were rinsed once with PBS and incubated with mixture reaction buffer (100 mmol/liter sodium ascorbate, 4.8 μmol/liter Alexa Fluor 488 azide, and 4 mmol/liter CuSO4) for 30 min at room temperature. After staining, cells were washed three times with 0.5% Triton X-100 in PBS, and DAPI was used for cell nuclei. Photos were taken by an Olympus IX81 inverted research microscope.

Statistical Analysis—All data are expressed as mean ± S.E. Two-group comparison was analyzed by Student's t test. p < 0.05 was considered significant.

Author Contributions—X. F., X. X., H. X. Y., and K. L. G designed the research, analyzed the data, and wrote the manuscript. X. F. performed the experiments with assistance from P. L., X. Z., M. T. L., F. L. I., Z. W., Z. P. M., and Y. P. S. Y. Y. provided technical and intellectual support. All authors discussed the results and commented on the manuscript.

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