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PP1 Cooperates with ASPP2 to Dephosphorylate and Activate TAZ*[S]

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The Hippo pathway regulates organ size by controlling both cell proliferation and apoptosis. TAZ functions as a transcriptional co-activator downstream of the Hippo pathway and has been implicated in human cancer development. A key step in the Hippo-TAZ pathway is phosphorylation of TAZ by LATS kinase, which leads to TAZ inhibition by both cytoplasmic retention and degradation. However, the mechanism of TAZ dephosphorylation and the responsible phosphatase are unknown. Here, we identified PP1 as a *bona fide* TAZ phosphatase. PP1A dephosphorylates TAZ at Ser-89 and Ser-311, promotes TAZ nuclear translocation, and stabilizes TAZ by disrupting the binding to the SCF E3 ubiquitin ligase. Furthermore, ASPP2 facilitates the interaction between TAZ and PP1 to promote TAZ dephosphorylation. As a result, PP1 and ASPP2 increase TAZ-dependent gene expression. This study demonstrates that PP1A and ASPP2 play a critical role in promoting TAZ function by antagonizing the LATS kinase through TAZ dephosphorylation.

Genetic screens in *Drosophila* have delineated a new tumor suppressor pathway, the Hippo pathway, which regulates organ size by controlling both cell proliferation and apoptosis (1). Both the components and functions of the Hippo pathway are conserved from *Drosophila* to mammals. In mammalian cells, LATS1/2 and MST1/2 are the homologs of *Drosophila* Warts and Hippo, respectively (2). YAP, the mammalian ortholog of *Drosophila* Yorkie, has been demonstrated to be phosphorylated and inhibited by LATS (3, 4). TAZ, first identified as a 14-3-3-binding protein, shares ~50% sequence identity with YAP and has also been shown to function as a transcriptional co-activator downstream of the Hippo pathway (5, 6). TAZ is involved in the development of multiple organs such as the lung, fat, muscle, bone, limb, and heart, as well as in many cellular, processes including stem cell differentiation, cell proliferation, and epithelial-mesenchymal transition (6–12). *Taz* knockout mice develop two severe abnormalities: polycystic kidney disease and emphysema (13, 14). Notably, elevated TAZ expression is observed in >20% of breast cancers, especially invasive ductal carcinomas (12).

We previously showed that the activity of TAZ is inhibited by LATS kinase in a mechanism similar to YAP regulation by the Hippo pathway (6). There are four H*X*R*XX*S LATS phosphorylation motifs in TAZ. We have demonstrated that phosphorylation of TAZ at Ser-89 by LATS promotes its interaction with 14-3-3, resulting in cytoplasmic sequestration and functional inhibition of TAZ. The S89A mutation makes TAZ partially resistant to inhibition by the Hippo pathway (6). In addition, TAZ stability is also regulated by LATS phosphorylation. A dual phosphorylation of TAZ by LATS and CK1 creates a binding site for β-TrCP (beta-Transducin repeat containing protein), the substrate-recruiting subunit of the $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligase (15). Phosphorylation of TAZ at Ser-311 by LATS provides the priming site for subsequent CK1 phosphorylation at Ser-314 in TAZ, therefore creating a functional phosphodegron for β-TrCP binding (15). Therefore, phosphorylation plays a major role in TAZ regulation in both subcellular localization and protein levels.

Protein phosphorylation is highly dynamic and reversible. This raises questions about whether and which protein phosphatases are involved in TAZ regulation. Ser/Thr protein phosphatases are classified into three families: PPM (metal-dependent protein phosphatases), PPP (phosphoprotein phosphatases), and FCP/SCP (TFIIF-associating component of RNA polymerase II CTD phosphatase/small) (16, 17). The PPP superfamily members, including PP1, PP2A (PP2), PP2B (PP3), and PP4–7, share the same catalytic mechanism (18). PP1 is a major eukaryotic Ser/Thr protein phosphatase involved in various cellular processes (19). Interestingly, in our previous TAZ affinity purification to identify TAZ-binding proteins (20), we also identified PP1A (a catalytic subunit of PP1) in the TAZ complex, indicating that PP1A is potentially TAZ phosphatase. Notably, PP1A has been reported to interact with ASPP2 (21), which is a member of the ASPP (ankyrin repeat-containing, SH3 domain-containing, and proline-rich

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region-containing protein) family, containing four ankyrin repeats, an SH3 domain, and a proline-rich region (22–24). Through C-terminal ankyrin repeats and the SH3 domain, ASPP2 has been shown to interact with numerous proteins, including p53 (25), Bcl-2 (26), RelA p65 (27), APC-2 (APCL) (28), YAP (29), and APP-BP1 (30). YAP, the mammalian homolog of TAZ, interacts with ASPP2 through the WW domain- and SH3 domain-binding motifs, but the biochemical and biological significance underlying the YAP-ASPP2 interaction is largely unknown.

In this stidy, we show that PP1A dephosphorylates TAZ at Ser-89 and Ser-311. By doing so, PP1A increases TAZ nuclear localization and protein levels. We also observed that ASPP2 facilitates the interaction between TAZ and PP1A, thus leading to TAZ dephosphorylation and induction of TAZ target gene expression. Our study identifies PP1A as a TAZ phosphatase and the function of ASPP2 and PP1A in TAZ regulation.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HEK293T cells and HeLa cells were cultured in DMEM (Invitrogen) supplemented with 10% FCS (HyClone), 100 units/ml penicillin, and streptomycin (Invitrogen). MCF10A cells were maintained in DMEM/F-12 medium (Invitrogen) supplemented with 5% horse serum (Invitrogen), 20 ng/ml EGF, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 100 ng/ml cholera toxin, 100 units/ml penicillin, and streptomycin (Invitrogen). Cell transfection was performed using Lipofectamine 2000 (Invitrogen) or the calcium phosphate method. Cells were harvested at 24 h post-transfection for protein analyses.

To establish stable TAZ-expressing and PP1α-expressing cells, pBABE-TAZ and pQCXIH-PP1α retroviruses were generated and used to infect MCF10A cells, and stable pools were selected in puromycin (1 μg/ml) and hygromycin (200 μg/ml), respectively.

Western Blot Analysis and Immunoprecipitation—Protein lysates were prepared by lysing 293T cells in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40, 1 mM EDTA, 1 mM PMSF, 25 mM NaF, and mixture protease inhibitors (Roche Applied Science). Cell lysate (40 μg) was resolved by SDS-PAGE, followed by Western blot analysis. Antibodies of FLAG (A00170, GenScript; or A2220, Sigma), HA (F7; sc7392, Santa Cruz Biotechnology), His (THE™ His tag antibody, GenScript), ASPP2 (611354, BD Biosciences), lamins A and C (A01455, GenScript), α-tubulin (RB-9281-P0, Lab Vision Corp.), and β-actin (13E5; 4970, Cell Signaling) were purchased commercially. Anti-phospho-Ser-311 and anti-phospho-Ser-89 TAZ antibodies were raised in rabbits using synthetic phosphopeptide as an antigen.

For immunoprecipitation experiments, 1 mg of cell lysate was incubated with anti-FLAG M2-agarose for 3 h at 4 °C. Beads were washed three times with lysis buffer and centrifuged at 5000 × *g* for 5 min between each wash. Protein was eluted from beads with 50 μl of Laemmli sample buffer (Bio-Rad). Lysates were resolved on 8 or 10% SDS-polyacrylamide gels and transferred onto nitrocellulose (Bio-Rad) for Western blotting.

Phosphatase Assay—PP1 (P0754S) was purchased from New England Biolabs. 293T cells were cotransfected with FLAG-TAZ and HA-LATS2. After 24 h of incubation, 293T cells were lysed with lysis buffer, and the cell lysate was incubated with anti-FLAG M2-agarose for 3 h at 4 °C. Beads were washed three times, first with lysis buffer and then with 1× NEBuffer for pyridoxamine phosphate supplemented with $MnCl_2$. FLAG-TAZ was eluted by 3×FLAG peptide in 1× NEBuffer for pyridoxamine phosphate supplemented with $MnCl_2$. The elution was divided equally, and an indicated amount of PP1 was added. After incubation for 30 min at 30 °C, the eluted lysates were added to SDS loading buffer and boiled at 99 °C. The phosphorylation level of FLAG-TAZ was determined by Western blotting.

Subcellular Fractionation—293T cells were washed with PBS and transferred to a 1.5-ml tube. The cells were lysed with Harvest buffer (10 mM HEPES (pH 7.9), 50 mM NaCl, 0.5 M sucrose, 0.1 mM EDTA, 0.5% Triton X-100, 1 mM DTT, 1 mM PMSF, 25 mM NaF, and mixture protease inhibitors). After incubation for 5 min on ice, the lysate was centrifuged at 1000 rpm for 10 min. The pellet (nuclear fraction) was washed three times with wash buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, and 0.1 mM EGTA) and then resuspended with 1× SDS loading buffer. The supernatant (cytoplasmic fraction) was transferred to a new tube and centrifuged at 14,000 rpm for another 15 min. The supernatant was transferred to a new tube and boiled in 1× SDS loading buffer.

RNA Isolation and Real-time PCR—Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen). cDNA was synthesized by reverse transcription using oligo(dT) as the primer, followed by real-time PCR with gene-specific primers in the presence of SYBR Premix Ex Taq (DRR041A, TaKaRa). The relative abundance of mRNA was calculated by normalization to GAPDH mRNA.

RESULTS

PP1A Is a TAZ Phosphatase—As a transcription co-activator, TAZ must interact with a downstream transcription factor to induce gene expression. In our previous work, we performed TAZ affinity purification to identify TAZ target transcription factors (20). Interestingly, this purification strategy identified multiple putative TAZ-interacting proteins, which included not only all four TEAD (TEA domain) family members but also PP1A. In fact, multiple peptides for the PP1A protein were identified (Fig. 1*A*). These peptides were absent from control purification of cells infected with the streptavidin-binding peptide vector. To confirm the interaction between TAZ and PP1A, we performed co-immunoprecipitation experiments and found that HA-PP1A could be readily pulled down by TAZ (Fig. 1*B*, *left panel*). Similarly, reciprocal co-immunoprecipitation experiments also showed that TAZ was co-precipitated with PP1A (Fig. 1*B*, *right panel*).

We recently characterized the conserved H*X*R*XX*S motifs in YAP and TAZ (3, 6). Both YAP and TAZ are phosphorylated by LATS kinase (3,6). However, the phosphatase responsible for dephosphorylation of both YAP and TAZ is unknown. The identification of PP1A in the TAZ purification

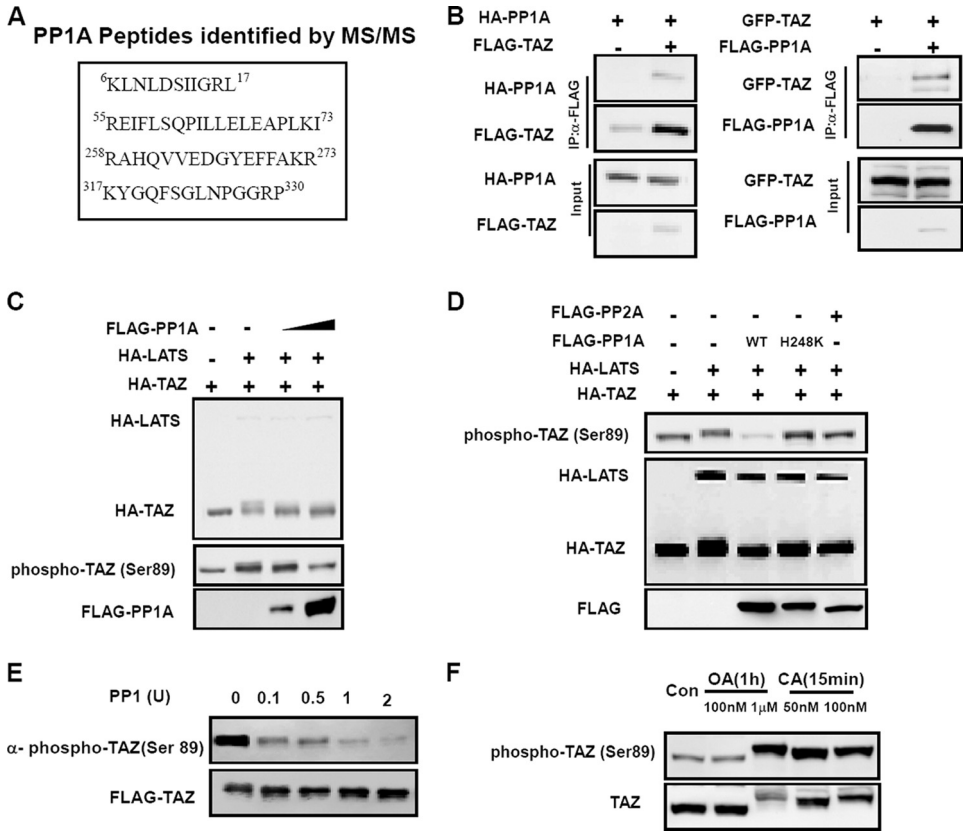

FIGURE 1. **PP1A dephosphorylates TAZ.** *A*, mass spectrometry identification of PP1A in FLAG-TAZ immunoprecipitation. Cell lysate from 293T cells stably expressing FLAG-TAZ was immunoprecipitated with streptavidin-binding peptide beads, followed by washing four times and trypsin digestion for further mass spectrometry analysis. The protein sequences of PP1A peptides identified by mass spectrometry are shown. *B*, TAZ binds to PP1A. HA-PP1A/FLAG-PP1A was cotransfected with FLAG-TAZ/GFP-TAZ into HEK293T cells as indicated. PP1A and TAZ associations were examined by reciprocal co-immunoprecipitation as indicated. *C*, PP1A dephosphorylates TAZ at Ser-89. The indicated plasmids were coexpressed in 293T cells, and TAZ phosphorylation at Ser-89 was determined by Western blotting. *D*, PP1 (but not PP2A) dephosphorylates TAZ at Ser-89. The indicated plasmids were coexpressed in 293T cells, and TAZ phosphorylation at Ser-89 was determined by Western blotting. H248K denotes the phosphatase-inactive mutant. *E*, PP1A dephosphorylates TAZ at Ser-89 *in vitro*. FLAG-TAZ was immunoprecipitated from transfected 293T cells and then incubated with the indicated amount of recombinant PP1. Phosphorylation of TAZ was detected by anti-phospho-Ser-89 TAZ antibody. *F*, phosphatase inhibitors induce TAZ phosphorylation at Ser-89. 293T cells were treated with phosphatase inhibitors as indicated, and TAZ phosphorylation at Ser-89 was determined by Western blotting. *OA*, okadaic acid; *CA*, calyculin A.

led us to test whether PP1A is a phosphatase for TAZ. To this end, we examined the effect of PP1A on TAZ phosphorylation at Ser-89. As shown in Fig. 1*C*, ectopic expression of LATS dramatically induced TAZ phosphorylation at Ser-89, but coexpression of PP1A significantly decreased TAZ phosphorylation at Ser-89 in a dose-dependent manner. PP1A belongs to the PPP family, which includes PP1 and PP2A. Recently, a PP2A complex has been reported to function as a negative regulator of the Hippo pathway in *Drosophila* (31). To identify whether the above observed dephosphorylation of TAZ is unique to PP1A, we also examined the effect of PP2A on TAZ phosphorylation at Ser-89. Interestingly, overexpression of only WT PP1A, but not the phosphatase-inactive mutant H248K or PP2A, dramatically decreased TAZ phosphorylation at Ser-89 (Fig. 1*D*). Furthermore, we performed an *in vitro* dephosphorylation assay and found that TAZ phosphorylation at Ser-89 was decreased with increasing amounts of PP1 (Fig. 1*E*). PP1A is inhibited by okadaic acid with an IC_{50} of 20 nM, whereas PP2A is much more sensitive to okadaic acid, with an IC_{50} of 0.2 nM (32). Calyculin A is a more efficient inhibitor for both PP1A and PP2A (33). Indeed, both okadaic acid and calyculin A significantly increased TAZ phosphorylation at Ser-89 and resulted in an obvious TAZ mobility shift as detected by the anti-TAZ antibody, indicating that endogenous TAZ is dynamically regulated by reversible phosphorylation (Fig. 1*F* and supplemental Fig. 1). The concentrations of okadaic acid and calyculin A needed to increase TAZ phosphorylation were consistent with PP1A as a physiological TAZ phosphatase.

PP1A Stabilizes TAZ by Decreasing the Interaction between TAZ and β-TrCP—Our recent work has shown that LATS and CK1 phosphorylate TAZ and promote TAZ interaction with the SCF E3 ubiquitin ligase and degradation (15). If PP1A dephosphorylates TAZ, it should stabilize TAZ by antagonizing the effect of LATS. To this end, we examined the effect of okadaic acid on TAZ protein levels. As shown in Fig. 2*A*, okadaic acid treatment resulted in a visible decrease in the TAZ protein level and increased TAZ phosphorylation. MG132 treatment blocked the effect of okadaic acid on TAZ protein levels, indicating that PP1A increases TAZ protein levels by suppressing proteasome-mediated degradation. To further characterize the PP1A effect on TAZ stability, we generated PP1A stable pools using a retrovirus. We observed an obvious increase in TAZ protein in pBABA-FLAG-TAZ/MCF10A cells stably expressing PP1A (Fig. 2*B*). Furthermore, the half-life of TAZ in the PP1A-expressing cells was significantly increased compared with that in the vector control cells (Fig. 2*C*).

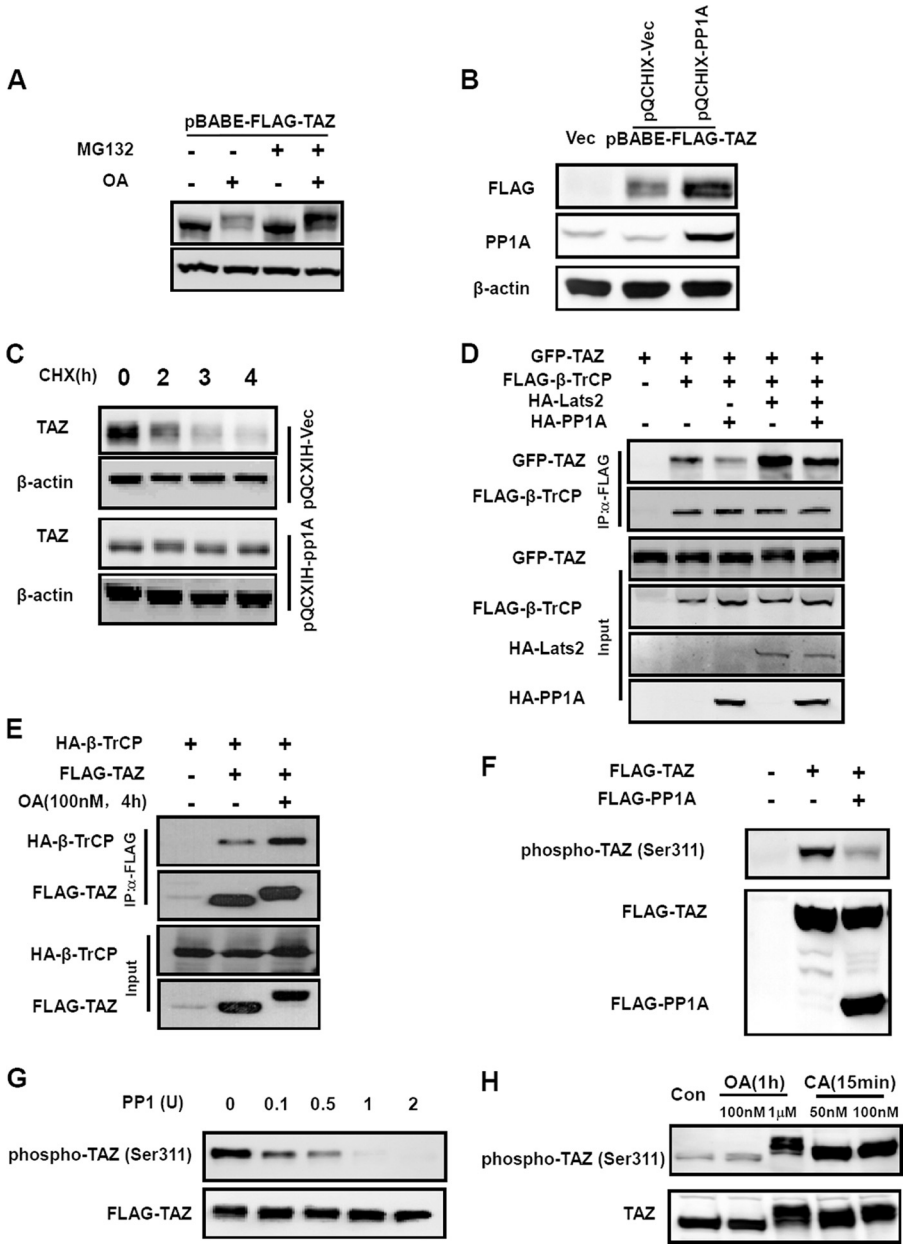

FIGURE 2. **PP1A stabilizes TAZ by decreasing the interaction between TAZ and β-TrCP.** *A*, phosphatase inhibitors promote proteasome-dependent TAZ degradation. 293T cells stably expressing TAZ were treated with okadaic acid (*OA*) and MG132 as indicated. The steady-state level of TAZ was determined by Western blotting. *B*, PP1A increases TAZ protein levels. Cell lysates from pBABE-FLAG-TAZ/MCF10A cells expressing the vector (*Vec*) or PP1A were separated and probed with the indicated antibodies. *C*, PP1A stabilizes TAZ. pBABE-FLAG-TAZ/MCF10A cells expressing the vector or PP1A were treated with cycloheximide (*CHX*; 20 μg/ml) for the indicated times. TAZ protein levels were determined by Western blotting. *D*, PP1A decreases the interaction between TAZ and β-TrCP. The indicated plasmids were cotransfected into 293T cells. FLAG-β-TrCP was immunoprecipitated with anti-FLAG antibody, and the co-precipitated GFP-TAZ was detected by TAZ Western blotting. *E*, okadaic acid treatment increases the interaction between TAZ and β-TrCP. The indicated plasmids were cotransfected into 293T cells. FLAG-TAZ was immunoprecipitated with anti-FLAG antibody, and the co-precipitated β-TrCP was detected by HA Western blotting. *F*, PP1A decreases TAZ phosphorylation at Ser-311. PP1A was coexpressed with TAZ in 293T cells, and TAZ phosphorylation at Ser-311 was determined by Western blotting. *G*, PP1A dephosphorylates TAZ at Ser-311 *in vitro*. FLAG-TAZ was immunoprecipitated from transfected 293T cells, followed by incubation with the indicated amount of recombinant PP1. Phosphorylation of TAZ was detected by anti-phospho-Ser-311 TAZ antibody. *H*, phosphatase inhibitors induce TAZ phosphorylation at Ser-311. 293T cells were treated with phosphatase inhibitors as indicated, and TAZ phosphorylation at Ser-311 was determined by Western blotting. *CA*, calyculin A.

Our recent study identified β-TrCP as a TAZ-binding protein to recruit the SCF E3 ubiquitin ligase (15). We then determined the effect of PP1A on the interaction between TAZ and β-TrCP. PP1A overexpression decreased the interaction between TAZ and β-TrCP in both the absence and presence of LATS coexpression (Fig. 2*D*). Furthermore, okadaic acid treatment increased TAZ phosphorylation and its interaction with β-TrCP (Fig. 2*E*). TAZ phosphorylation at Ser-311 is required for C-terminal phosphodegron-mediated degradation (15). We then determined the effect of PP1A on TAZ phosphorylation at Ser-311. We observed a dramatically decreased TAZ phosphorylation at Ser-311 in the presence of PP1A (Fig. 2*F*). As expected, PP1A efficiently dephosphorylated TAZ at Ser-311 *in vitro* (Fig. 2*G*). Con-

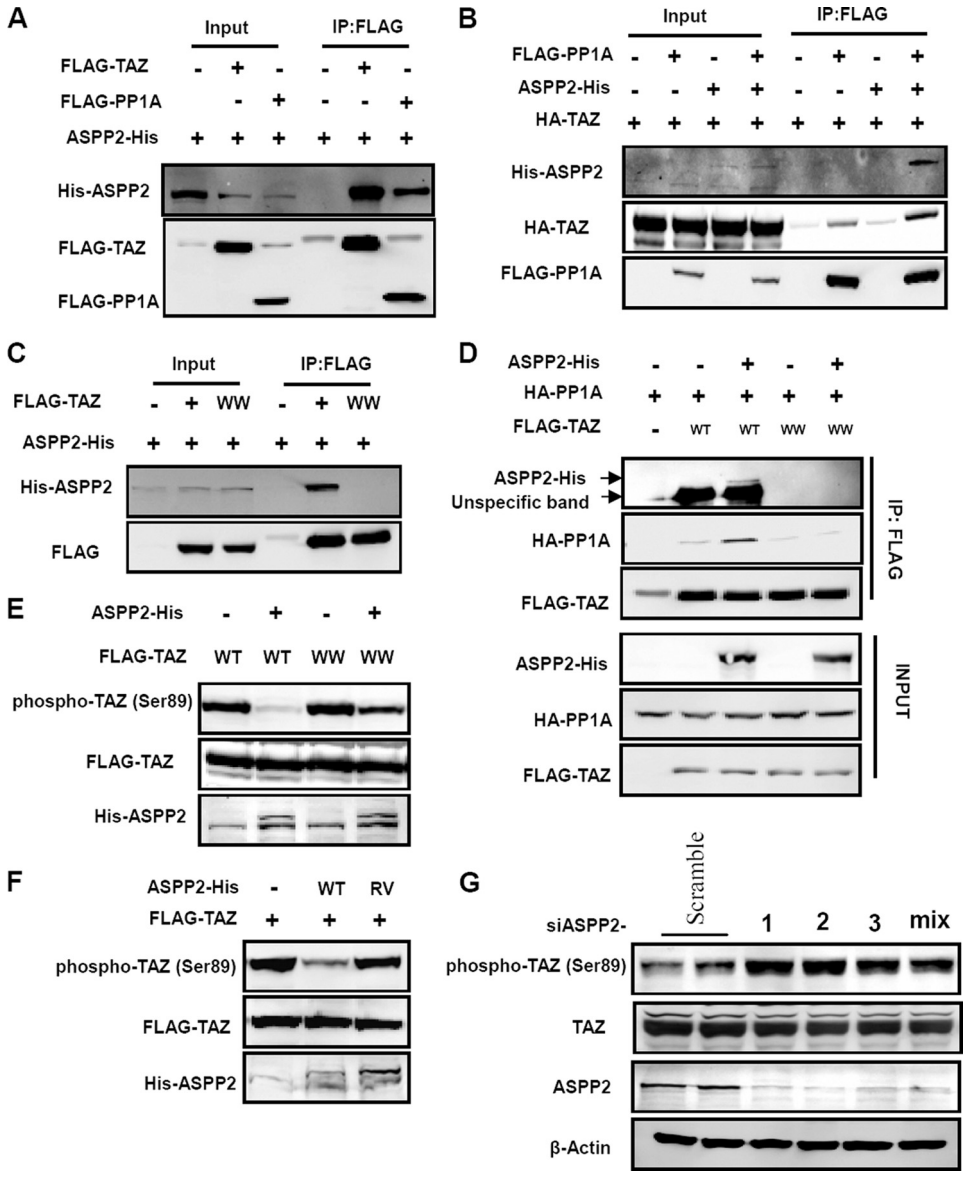

FIGURE 3. **ASPP2 increases the interaction between TAZ and PP1A.** *A*, both TAZ and PP1A interact with ASPP2. ASPP2 plasmids were cotransfected into 293T cells as indicated. FLAG-TAZ or FLAG-PP1A was immunoprecipitated (*IP*) with anti-FLAG antibody, and the co-precipitated ASPP2-His was detected by His Western blotting. *B*, ASPP2 facilitates the interaction between TAZ and PP1A. The indicated plasmids were cotransfected into 293T cells. FLAG-PP1A was immunoprecipitated with anti-FLAG antibody, and the co-precipitated HA-TAZ or ASPP2-His was detected by HA or His Western blotting. *C*, TAZ requires its WW domain to interact with ASPP2. The indicated plasmids were cotransfected into 293T cells. FLAG-TAZ was immunoprecipitated with anti-FLAG antibody, and the co-precipitated ASPP2-His was detected by His Western blotting. *D*, the WW domain in TAZ is required for ASPP2 to enhance the interaction between TAZ and PP1A. The indicated plasmids were cotransfected into 293T cells. FLAG-TAZ was immunoprecipitated with anti-FLAG antibody, and the co-precipitated ASPP2-His or HA-PP1A was detected by His or HA Western blotting. *E*, the WW domain in TAZ is important for its dephosphorylation by ASPP2. The indicated plasmids were coexpressed in 293T cells, and TAZ phosphorylation at Ser-89 was determined by Western blotting. *F*, the PP1-binding motif RV*X*F in ASPP2 is required for it to promote dephosphorylation of TAZ Ser-89. The indicated plasmids were coexpressed in 293T cells, and TAZ phosphorylation at Ser-89 was determined by Western blotting. *G*, ASPP2 knockdown increases TAZ phosphorylation at Ser-89. Three ASPP2 RNA interference oligonucleotides were individually or in combination transfected into A375 cells as indicated. Levels of ASPP2 and TAZ phosphorylation at Ser-89 were determined by Western blotting.

sistently, both okadaic acid and calyculin A significantly increased TAZ phosphorylation at Ser-311 (Fig. 2*H*). These results demonstrate that PP1A stabilizes TAZ by dephosphorylating TAZ at Ser-311 and decreasing its interaction with β-TrCP.

ASPP2 Increases the Interaction between TAZ and PP1A to Promote TAZ Dephosphorylation—ASPP2 has been reported to interact with multiple proteins, including PP1 (21–24). It has also been reported to interact with the TAZ paralog YAP (29). Indeed, ASPP2 was identified as a TAZ-interacting protein with a high score in our TAZ-streptavidin-binding peptide tandem mass spectrometry experiment (supplemental Fig. 2). Therefore, we speculated whether ASPP2 may facilitate the interaction between TAZ and PP1. To explore the functional role of ASPP2 in TAZ dephosphorylation via PP1, we first tested whether TAZ and PP1A can interact with ASPP2. Both TAZ and PP1A could readily pull down ASPP2 (Fig. 3*A*). We also found that ASPP2 increased the interaction between TAZ and PP1A (Fig. 3*B*). Furthermore, the TAZ WW mutant abolished its interaction with ASPP2 (Fig. 3*C*),

similar to a previous report that YAP interacts with ASPP2 via its WW1 domain (29). In addition, ASPP2 increased the interaction between WT TAZ and PP1A but had no promoting effect on TAZ WW mutant interaction with PP1A (Fig. 3*D*). Moreover, ASPP2 overexpression dramatically decreased WT TAZ phosphorylation at Ser-89 but had less of an effect on TAZ WW mutants (Fig. 3*E* and supplemental Fig. 3). The above results suggest that ASPP2 may stimulate TAZ dephosphorylation partly by promoting the interaction between TAZ and PP1 and that this function of ASPP2 requires the TAZ WW domain.

It has been reported that a short peptide from ASPP2 containing the PP1-binding motif (RV*X*F) interacts with the catalytic subunit of PP1 (34). We then tested whether the ability to interact with PP1A is required for ASPP2 to promote TAZ dephosphorylation. As shown in Fig. 3*F*, WT ASPP2 overexpression dramatically decreased TAZ phosphorylation at Ser-89, whereas the PP1-interacting defective RV mutant, in which the arginine and valine in the RV*X*F motif were replaced by glutamate and aspartate, totally blocked this effect. In contrast, ASPP2 (but not scrambled) RNAi interference oligonucleotides significantly increased TAZ phosphorylation at Ser-89 (Fig. 3*G*). These data support a role of endogenous ankyrin repeat-containing, SH3 domain-containing, and proline-rich region-containing protein 2 in promoting TAZ dephosphorylation and a potential mechanism by increasing the interaction between TAZ and PP1A.

PP1A and ASPP2 Promote TAZ Nuclear Localization—TAZ phosphorylation at Ser-89 creates a 14-3-3-binding site that results in cytoplasmic retention. This suggests that TAZ dephosphorylation by PP1A could affect its interaction with 14-3-3 and subcellular localization. To this end, we examined the binding between TAZ and 14-3-3 with or without PP1A coexpression. We observed that PP1A overexpression significantly decreased the binding between TAZ and 14-3-3 (Fig. 4*A*). Consistently, phosphatase inhibitor calyculin A and okadaic acid treatment increased the interaction between TAZ and 14-3-3 (Fig. 4*B*). We also observed that coexpression of WT ASPP2 significantly decreased the interaction between TAZ and 14-3-3. In contrast, the RV mutant was totally inactive in affecting the binding between TAZ and 14-3-3 (Fig. 4*C*). Furthermore, PP1A overexpression resulted in a dramatic increase in TAZ nuclear localization, and ASPP2 had a similar effect (Fig. 4*D*). Moreover, okadaic acid treatment led to TAZ cytoplasmic retention (Fig. 4*E*). Collectively, our data suggest that PP1A and ASPP2 promote TAZ nuclear localization via dephosphorylation.

PP1A and ASPP2 Regulate TAZ Target Gene Expression—TAZ is known to induce epithelial-mesenchymal transition and promote cell proliferation (6, 12). As a transcription coactivator, TAZ exerts its biological function by modulating gene expression. To determine the functional significance of PP1A and ASPP2 in TAZ regulation, we performed quantitative PCR to examine the PP1A and ASPP2 effect on epithelial-mesenchymal transition markers and cell growth markers, which are regulated by TAZ. As shown in Fig. 5*A*, PP1A overexpression significantly enhanced the ability of TAZ to induce N-cadherin, a marker for mesenchymal cells, and to reduce E-cadherin, a marker for epithelial cells, as determined in MCF10A cells stably expressing TAZ and PP1A. Similarly, coexpression of PP1A increased the TAZ-induced expression of CTGF,[3] which is a TAZ target gene involved in promoting cell growth (Fig. 5*A*). Furthermore, ASPP2 knockdown significantly decreased CTGF expression in MCF10A cells stably expressing TAZ but had a much weaker effect in cells stably expressing TAZ(4SA), which is a constitutively active mutant no longer inhibited by phosphorylation (Fig. 5*B*). Collectively, the above data support that PP1 and ankyrin repeat-containing, SH3 domain-containing, and proline-rich region-containing protein 2 activate TAZ as indicated by the increase of TAZ-dependent gene expression. Moreover, the effect of PP1 and ASPP2 on TAZ target gene expression appears to be mediated via TAZ dephosphorylation.

DISCUSSION

In this work, we have shown that both PP1A and ASPP2 promote TAZ dephosphorylation, therefore resulting in TAZ nuclear retention and induction of TAZ target gene expression. Our study reveals a molecular mechanism of dynamic reversible phosphorylation in regulation of TAZ by the Hippo pathway and PP1A.

We have previously demonstrated that phosphorylation negatively regulates TAZ by two mechanisms. Phosphorylation of Ser-89 inhibits TAZ by cytoplasmic sequestration, whereas phosphorylation of Ser-311 inactivates TAZ by promoting ubiquitination and degradation (15). Therefore, phosphorylation plays a major role in TAZ regulation. In this study, we have shown that TAZ phosphorylation is also controlled by PP1A and its interacting protein ASPP2. Several lines of evidence support a role of PP1A in TAZ dephosphorylation. First, PP1A is identified in the TAZ complex, and the interaction between TAZ and PP1A is confirmed by reciprocal co-immunoprecipitation. The PP1-interacting protein ASPP2 is also consistently identified in the TAZ complex. Second, coexpression of PP1A reduces the TAZ mobility shift and decreases TAZ phosphorylation at Ser-89 and Ser-311, as indicated by Western blotting with the anti-phospho-Ser-89 TAZ and anti-phospho-Ser-311 TAZ antibodies. Third, both phosphatase inhibitors okadaic acid and calyculin A increase TAZ phosphorylation at Ser-89 and Ser-311. The concentration of okadaic acid needed to increase TAZ phosphorylation is consistent with PP1A being the target phosphatase. Fourth, PP1 efficiently dephosphorylates both Ser-89 and Ser-311 in TAZ by *in vitro* reactions. Furthermore, coexpression of PP1A promotes TAZ nuclear localization and TAZ stabilization. Interestingly, YAP dephosphorylation is not similarly controlled by PP1A (supplemental Fig. 4). It is worth the effort to identify the phosphatase responsible for YAP dephosphorylation.

Previously, ASPP2 was identified as a PP1-interacting protein, but the biological function of this interaction remained to be explored. Here, we have shown that ASPP2

[3] The abbreviations used are: CTGF, connective tissue growth factor; β-TrCP, beta-transducin repeat containing protein.

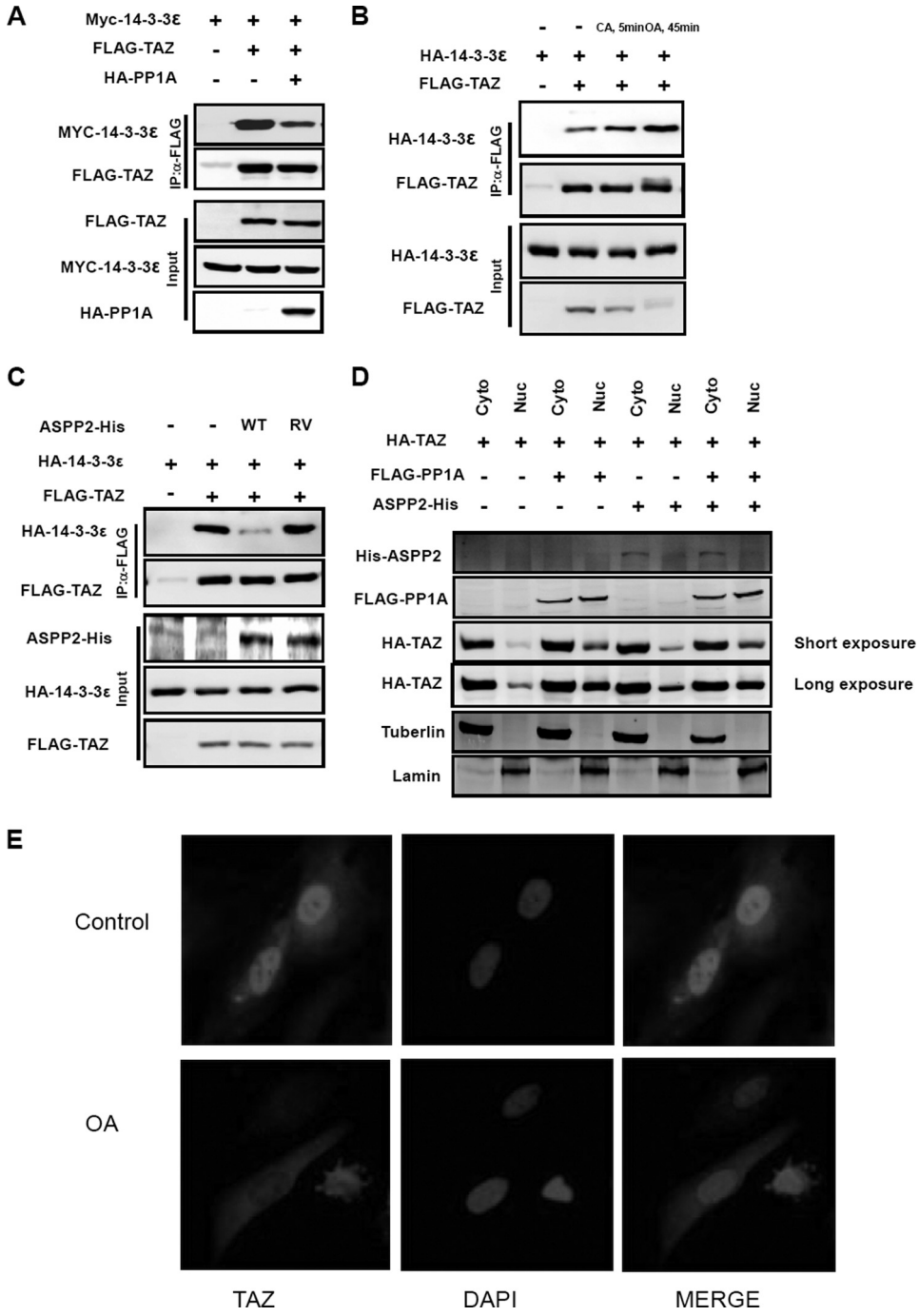

FIGURE 4. **PP1A and ASPP2 promote TAZ nuclear localization.** *A*, PP1A decreases the interaction between TAZ and 14-3-3. The plasmids were cotransfected into 293T cells as indicated. FLAG-TAZ was immunoprecipitated (*IP*) with anti-FLAG antibody, and the co-precipitated Myc-14-3-3 was detected by Myc Western blotting. *B*, phosphatase inhibitors increase the interaction between TAZ and 14-3-3. The plasmids were cotransfected into 293T cells as indicated. FLAG-TAZ was immunoprecipitated with anti-FLAG antibody, and the co-precipitated HA-14-3-3 was detected by HA Western blotting. *CA*, calyculin A. *C*, the PP1-binding motif RV*X*F is required for ASPP2 to inhibit the interaction between TAZ and 14-3-3. The plasmids were cotransfected into 293T cells as indicated. FLAG-TAZ was immunoprecipitated with anti-FLAG antibody, and the co-precipitated HA-14-3-3 was detected by HA Western blotting. *D*, PP1A and ASPP2 promote TAZ nuclear localization. The plasmids were cotransfected into 293T cells as indicated. Cytoplasmic (*Cyto*) and nuclear (*Nuc*) fractions were separated for Western blot analysis as indicated. *E*, phosphatase inhibitor treatment increases the TAZ cytoplasmic sequester. BT549 cells were treated with 100 nM okadaic acid (*OA*) for 2 h. Immunofluorescence was performed to determine TAZ cytoplasmic/nuclear distribution with anti-TAZ antibody (BD Biosciences).

promotes TAZ-PP1 interaction by binding with both TAZ and PP1 through different domains, thus enhancing dephosphorylation of TAZ. Interestingly, although the WW domain mutant significantly abolishes the interaction between TAZ and ASPP2, ASPP2 still enhances dephosphorylation of TAZ, indicating another indirect effect on TAZ. One possibility is that ASPP2 can regulate insulin receptor substrate 1 (35), which has been found to bind with YAP in the cytoplasm (36), to affect the YAP/TAZ subcellular location and phosphorylation level.

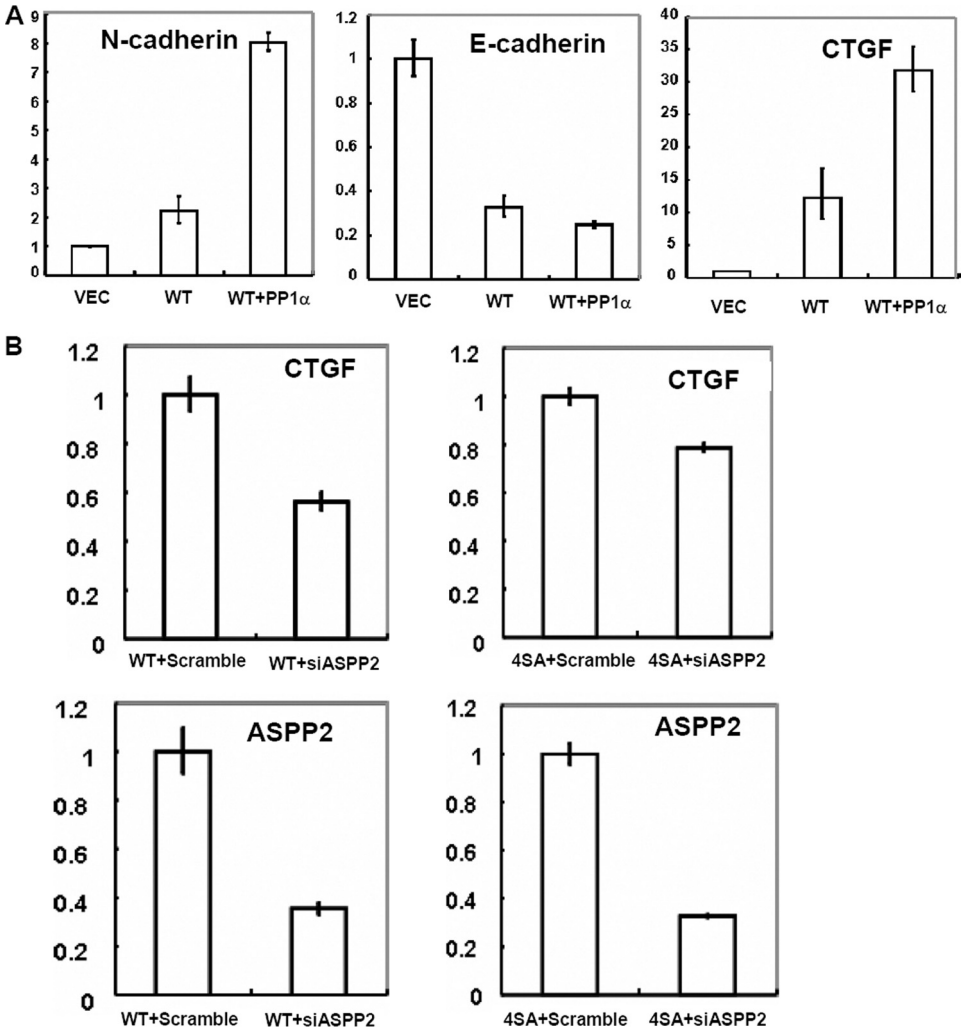

FIGURE 5. **PP1A and ASPP2 promote TAZ target gene expression.** *A*, PP1A increases N-cadherin and CTGF expression and decreases E-cadherin expression in pBABE-FLAG-TAZ/MCF10A cells. Total RNA was extracted from vector-expressing, pBABE-FLAG-TAZ/pQCXIH-vector-, and pBABE-FLAG-TAZ/pQCXIH-PP1A-expressing MCF10A cells, and quantitative PCR was performed to determine E-cadherin, N-cadherin, and CTGF expression. All data are normalized to GAPDH. *B*, ASPP2 knockdown decreases CTGF expression. ASPP2 was depleted in WT TAZ and TAZ(4SA)-expressing MCF10A cells using siRNA against human ASPP2 (*siASPP2*). The efficiency of ASPP2 knockdown and down-regulation of CTGF was determined by relative quantitative PCR.

ASPP2 is a haploinsufficient tumor suppressor (37), and it promotes the apoptosis function of p53, similar to p63 and p73 (22). Besides the p53 family, ASPP2 interacts with numerous proteins, including YAP. However, the biological function of the interaction between ankyrin repeat-containing, SH3 domain-containing, and proline-rich region-containing protein 2 and non-p53 families is largely unknown. The interaction between ASPP2 and APP-BP1 inhibits apoptosis in neuronal cells, indicating that ASPP2 could have an anti-apoptotic function (30). Here, we have shown that ASPP2 activates TAZ, also indicating an anti-apoptotic function because TAZ inhibits apoptosis. These studies suggest that the biological significance of ASPP2 in cooperating with non-p53 families is cell context-dependent.

ASPP2 interacts with TAZ and PP1A via the PY motif and RV*X*F motif, respectively. ASPP2 belongs to the ASPP family, including pro-apoptotic ASPP1 and anti-apoptotic iASPP (22–24). ASPP1, but not iASPP, also possesses a PY motif and an RV*X*F motif, implying that ASPP1 may also regulate the TAZ phosphorylation level in a manner similar to ASPP2.

TAZ has also been implicated in human tumorigenesis. TAZ is inhibited by the Hippo pathway, which contains the well established human tumor suppressor NF2 (6) and WW45 and Mob, which are mutated in human cancer cell lines (38, 39). In addition, overexpression of TAZ in MCF10A cells can promote cell proliferation, epithelial-mesenchymal transition, and oncogenesis (6, 12, 20). We previously demonstrated that LATS phosphorylates TAZ to promote its ubiquitin-mediated degradation, leading to functional inhibition of TAZ (15). Notably, elevated TAZ expression is observed in >20% of breast cancers, especially invasive ductal carcinomas (12). Here, we demonstrated that PP1A coupled with ASPP2 antagonizes the function of LATS to regulate the reversible phosphorylation of TAZ. Given the potential role of TAZ in human cancer, it would be interesting to examine the expression of PP1 and ASPP2 in TAZ-overexpressed breast cancer tissues. Our studies shed light on the dynamic regulation of TAZ phosphoryla-

tion and potential mechanisms of TAZ activation in human cancer.

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Signal Transduction:

PP1 Cooperates with ASPP2 to Dephosphorylate and Activate TAZ

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