Mst2 and Lats Kinases Regulate Apoptotic Function of Yes Kinase-associated Protein (YAP)*

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The Hippo pathway in Drosophila controls the size and shape of organs. In the fly, activation of this pathway conveys growth-inhibitory signals and promotes apoptosis in epithelial cells. We “reconstituted” the Hippo pathway in a human epithelial cell line and showed that, in contrast to flies, the activation of this pathway results in anti-apoptotic signals. We have shown that in human embryonic kidney (HEK) 293 cells, the complex formation between transcriptional co-activators YAPs (Yes kinase-associated proteins) and Lats kinases requires the intact WW domains of YAPs, as well as intact Pro-Pro-AA-Tyr (where AA is any amino acid) motifs in Lats kinases. These kinases cooperate with the upstream Mst2 kinase to phosphorylate YAPs at Ser-127. Overexpression of YAP2 in HEK293 cells promoted apoptosis, whereas the Mst2/Lats1-induced phosphorylation of YAP partially rescued the cells from apoptotic death. Apoptotic signaling of YAP2 was mediated via stabilization of p73, which formed a complex with YAP2. All components of the Hippo pathway that we studied were localized in the cytoplasm, with the exception of YAP, which also localized in the nucleus. The localization of YAP2 in the nucleus was negatively controlled by the Lats1 kinase. Our apoptotic “readout” of the Hippo pathway in embryonic kidney cells represents a useful experimental system for the identification of the putative upstream receptor, membrane protein, or extracellular factor that initiates an entire signaling cascade and ultimately controls the size of organs.

Metazoans have evolved several well conserved signaling pathways that regulate cell proliferation and growth to create organs, and ultimately organisms, of reproducible size and shape (1). One such pathway, known as the Hippo (Hpo) pathway, was originally identified in Drosophila melanogaster (2–11). Flies with “loss-of-function” mutations in components of the Hpo pathway develop significantly overgrown organs. Conversely, activation of this pathway results in reduced proliferation of cells and increased sensitivity to developmentally regulated apoptosis (12). Several proteins constitute the core of the Hpo pathway; these include a scaffolding protein, two serine/threonine kinases, and one transcriptional co-activator (13). The most well characterized upstream signaling component of the pathway is Salvador (Sav), a gene product that contains two protein-protein interaction modules known as WW domains (14) and is believed to act as a scaffolding protein for Hpo and Warts (Wts) (15, 16). Hpo is a serine/threonine protein kinase that phosphorylates and activates Wts (17, 18). At the amino-terminal portion of the protein, Wts has five PPXY motifs (where X represents any amino acid), which are binding sites to class I WW domains (19). Hpo-activated Wts binds and phosphorylates Yki, subsequently preventing Yki from stimulating the transcription of diap1 and CycE genes, which ultimately results in reduced cell proliferation (12). Wts, Hpo, and Sav are negative regulators of Yki, as the Yki overexpression phenotype resembles that of the functional loss of these three proteins (12, 20).

The human orthologs of the Hpo pathway are well conserved (Fig. 1A). The most upstream is WW45 (WW domain-containing protein, 45-kDa molecular mass), the ortholog of Sav, which also contains two WW domains (15). From this point, the pathway bifurcates. The remaining downstream components of the Hpo pathway in humans are represented by two closely related paralogs. Mst1 and Mst2 (mammalian Ste20-like protein kinases) are the mammalian orthologs of Hpo and are closely related serine/threonine kinases known to phosphorylate large tumor suppressor kinases, Lats1 and Lats2 (two serine/threonine kinases that are orthologs of Wts) (21–26). Phosphorylated Lats1 and Lats2 display enhanced kinase activity (27). Similar to Wts, Lats1 and Lats2 have PPXY sequence motifs at their amino-terminal regions. The human ortholog of Drosophila Yki, YAP, also exists as two splice isoforms: YAPI, which contains one WW domain, and YAP2, which contains two WW domains (28, 29). YAP is a transcriptional co-activator in which function depends on the presence of an intact WW domain(s) (29, 30).

We decided to study the YAP signaling function in the context of the Hpo pathway in human cells for several reasons. The...
first thing to catch our attention was the apparent paradox of YAP functioning either as an oncogene (31–34) or a promoter of apoptosis (35–39). Secondly, the Hpo pathway is an ideal pathway for studying signaling mediated by WW domains because all components of the main pathway contain either WW domains or their cognate ligand motifs, PPXY (14, 40) or PPXF (41) (Fig. 1A). Thirdly, the protein module known as the WW domain and its founding protein, YAP, are the main focus of research in our laboratory. Using our expertise and reagents, we have concentrated on the critical role of the WW domain in mediating multiple, yet specific protein-protein interactions that occur in the Hpo pathway.

We have shown that in the human epithelial cell line HEK293, the complex formation of YAP1 and YAP2 with Lats1 and Lats2 requires intact WW domain(s) in YAPs as well as intact PPXY motifs in Lats kinases. These kinases cooperate with the Mst2 kinase to phosphorylate YAP at Ser-127. Over-expression of YAP2 in HEK293 cells promotes apoptosis of HEK293 cells, whereas the Mst2/Lats1-induced phosphorylation of YAP partially rescues cells from apoptosis. The apoptotic signaling of YAP2 is mediated via stabilization of p73, which forms a complex with both of the WW domains of YAP2. Our data provide evidence of the proapoptotic function of YAP and contradict that of Yki, the fly homolog of YAP, which has been shown to act as an oncogene (12).

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—HEK293 and NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were transiently transfected using Lipofectamine (Invitrogen). For the immunoprecipitation of YAP, protein A-Sepharose (Sigma) was incubated with anti-YAP antibody or control IgG against human YAP was generated in rabbits. The animals were injected with purified antigen that contained GST fused to amino acids 302–450 of human YAP1 (GenBank NP 006097 28) expressed in bacteria using pGEX-2TK vector. GST-YAP fusion protein was purified to homogeneity using glutathione-Sepharose chromatography. Immune sera were partially depleted of antibodies reacting with GST by affinity chromatography on GST-Sepharose. The sera were then affinity-purified on GST-YAP antigen coupled to Sepharose, concentrated by ammonium sulfate precipitation, and dialyzed against phosphate-buffered saline solution (42). The high titer of the preparation was represented by positive Western blotting data obtained at a 1:20,000 dilution of the antibody.

**Immunoprecipitation**—HEK293 cells were transfected with expression vectors that encoded the protein of interest using Lipofectamine (Invitrogen). 24 h later, cells were lysed with modified radioimmune precipitation assay buffer (50 mM Tris-HCl (pH 7.45), 5 mM EDTA, 300 mM NaCl, 1% glycerol, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) and immunoprecipitated using anti-FLAG M2 antibody (Sigma) and FLAG-M2 antibody were from Sigma. Polyclonal antibody against human YAP was generated in rabbits. The animals were injected with purified antigen that contained GST fused to amino acids 302–450 of human YAP1 (GenBank NP 006097 28) expressed in bacteria using pGEX-2TK vector. GST-YAP fusion protein was purified to homogeneity using glutathione-Sepharose chromatography. Immune sera were partially depleted of antibodies reacting with GST by affinity chromatography on GST-Sepharose. The sera were then affinity-purified on GST-YAP antigen coupled to Sepharose, concentrated by ammonium sulfate precipitation, and dialyzed against phosphate-buffered saline solution (42). The high titer of the preparation was represented by positive Western blotting data obtained at a 1:20,000 dilution of the antibody.

**Establishment of Cell Lines That Express YAP in an Inducible System**—HEK293 cells were transfected with pcDNA6/TR vector (Invitrogen), which encodes the Tet repressor under the control of the human cytomegalovirus promoter. Cells that failed to express this repressor were removed by adding blastcidin to a final concentration of 5 μg/ml. After the selection, cells were transfected with YAP cDNAs that were cloned into pcDNA4/TO/myc-His vector (Invitrogen). Selection was performed by adding both blastcidin (5 μg/ml) and zeocin (400 μg/ml). Single-cell isolation was never performed when cells were selected. After the establishment of the cell lines, the concentration of zeocin was reduced to 200 μg/ml, and that of blastcidin remained at 5 μg/ml. Tetracycline was added to the medium to a final concentration of 1 μg/ml to induce the expression of YAPs. The same protocol was used for NIH3T3 cells, except higher concentrations of blastcidin (10 μg/ml) were used in the selection of transformants.

**Cell Counting Assay**—293 cells were distributed in DMEM containing 1% FBS, blastcidin (5 μg/ml), and zeocin (200 μg/ml) at an initial density of about 2–3%. 12 h later, tetracycline (1 μg/ml) was added to the medium to induce the expression of YAPs. 96 h later, floating cells were removed, and attached cells were tryspinized and counted. For NIH3T3 cells,
more FBS and blasticidin were added to the medium up to 2.5% and 10 μg/ml, respectively.

**RNAi Treatment**—The RNAi oligos for p73 and YAP were purchased from Dharmacon and Santa Cruz Biotechnology, respectively. Luciferase-specific RNAi oligo was used as a control. The target sequence for p73 is CCAUCCUGUACACUCUAUGU. HEK293 cells that express YAP2 WT in an inducible system or normal HEK293 cells were plated at ~25% confluency. Transfections of the RNAi oligos were conducted by using Lipofectamine RNAiMAX (Invitrogen). Cells were incubated for 48 h, and the amount of endogenous p73 and YAP was determined by Western blotting.

**RESULTS**

**YAP Binds Preferentially to Both Lats1 and Lats2**—Because all components of the Hpo pathway in human contain either a PPX/PPXF motif present in individual proteins is also shown. Note that recently an isoform of Yki that contains one WW domain was identified. B, YAP2 forms a complex with Lats1 and Lats2. HEK293 cells were transiently co-transfected with YAP2 and various constructs expressing FLAG-tagged orthologs of the Hippo-Salvador pathway (p2xFlag-CMV2 vector as a control, WW45, Mst2, Lats1, and Lats2). Cell lysates were immunoprecipitated with FLAG antibodies, resolved on SDS-PAGE, and immunoblotted with YAP antibody (upper panel). The middle and lower panels show the expression of transfected proteins. C, endogenous YAP binds to endogenous Lats1 in HEK293 cells. HEK293 cell lysates were incubated with protein A-Sepharose conjugated with either control IgG or YAP antibody. The immunoprecipitates (IP) were washed, and bound proteins were separated by SDS-PAGE followed by immunoblotting (IB). D, intact PPXY motifs in Lats1 are required for binding to YAP2. Two PPXY sequences in Lats1 were mutated to PPPA. WT or single (PY1* or PY2*) or double (PY1* & PY2*) mutants of Lats1 in the FLAG tag vector were transiently co-transfected with YAP2 (in pcDNA4/HisMax) into HEK293 cells. Cells were lysed and analyzed as described in B. E, intact WW domains in YAP2 are required for binding to Lats1. Two WW domains in YAP2 were mutated to render them inactive in terms of ligand binding. WT or single (1st WW* or 2nd WW*) or double (1&2 WW*) mutant of YAP2 in pcDNA4/HisMax vector were co-transfected with FLAG-Lats1 or FLAG vector alone. Cells were lysed and analyzed as described in E.

**FIGURE 1.** YAP binds to Lats1 and Lats2 through the WW domain-PPXY complex. A, diagram comparing the components of the Hippo pathway in Drosophila with their human orthologs. The percentage of sequence similarity between individual orthologs was derived from DDBJ-ClustalW comparisons. The number of WW domains and PPXY/PPXF motifs present in individual proteins is also shown. Note that recently an isoform of Yki that contains one WW domain was identified. B, YAP2 forms a complex with Lats1 and Lats2. HEK293 cells were transiently co-transfected with YAP2 and various constructs expressing FLAG-tagged orthologs of the Hippo-Salvador pathway (p2xFlag-CMV2 vector as a control, WW45, Mst2, Lats1, and Lats2). Cell lysates were immunoprecipitated with FLAG antibodies, resolved on SDS-PAGE, and immunoblotted with YAP antibody (upper panel). The middle and lower panels show the expression of transfected proteins. C, endogenous YAP binds to endogenous Lats1 in HEK293 cells. HEK293 cell lysates were incubated with protein A-Sepharose conjugated with either control IgG or YAP antibody. The immunoprecipitates (IP) were washed, and bound proteins were separated by SDS-PAGE followed by immunoblotting (IB). D, intact PPXY motifs in Lats1 are required for binding to YAP2. Two PPXY sequences in Lats1 were mutated to PPPA. WT or single (PY1* or PY2*) or double (PY1* & PY2*) mutants of Lats1 in the FLAG tag vector were transiently co-transfected with YAP2 (in pcDNA4/HisMax) into HEK293 cells. Cells were lysed and analyzed as described in B. E, intact WW domains in YAP2 are required for binding to Lats1. Two WW domains in YAP2 were mutated to render them inactive in terms of ligand binding. WT or single (1st WW* or 2nd WW*) or double (1&2 WW*) mutant of YAP2 in pcDNA4/HisMax vector were co-transfected with FLAG-Lats1 or FLAG vector alone. Cells were lysed and analyzed as described in E.
WW domain complex. To test this hypothesis, we mutated two PPXY sequences in Lats1 (first, PPXY amino acids 373–376, and second, PPXY amino acids 556–559) by substituting the terminal Tyr for Ala. Such a mutation has been known to abrogate binding to WW domains (29, 43). The mutants of the PPXY cores (named PY1* and PY2*) and double mutant (PY1*&2*) were constructed. All three mutants, as well as Lats1 WY, were fused to FLAG tags and transiently co-expressed with YAP2 in HEK293 cells following by immunoprecipitation and immunoblotting (Fig. 1D). Lats1 WT bound strongly to YAP2 (Fig. 1D, upper panel). As anticipated, binding became weaker when either of the single mutants (PY1* or PY2*) was analyzed. When both PPXY motifs in Lats1 were mutated (PY1* and PY2*), the complex with YAP2 was abrogated. These data suggest that both PPXY sequences in Lats1 are necessary for full-strength association with YAP2.

We then asked whether either one or both WW domains in YAP2 mediate the complex with Lats1. To test this query, point mutations were introduced into the WW domains of YAP2. Two highly conserved amino acids in each of the two WW domains, the second signature Trp and the carboxy-terminal Pro, were mutated to Ala. The WQDP sequence of amino acids 199–202 in the first WW domain of YAP2 was changed to AQDA, whereas the WLDP sequence of amino acids 258–261 in the second WW domain of YAP2 was changed to ALDA. Such a substitution in WW domains renders the mutated domains inactive in terms of ligand binding (29). The mutants were named 1st WW* and 2nd WW*, respectively. A double mutant was named 1&2 WW*. All three YAP2 mutants and the WT were co-transfected with FLAG-Lats1 into HEK293 cells followed by immunoprecipitation and immunoblotting (Fig. 1E). Relatively strong binding was detected in the case of WT YAP2 (Fig. 1E, upper panel); however, this binding was weakened when the first WW domain of YAP2 was mutated. The second WW domain mutant seemed to have no influence on binding. Finally, when both WW domains of YAP2 were mutated, the binding was barely detectable. Similar results were obtained for Lats2 (data not shown). These results suggest that both WW domains in YAP2 play a crucial role in binding to Lats1 and Lats2 and that the first WW domain plays a more dominant role in the complex. Interestingly, although the first WW domain of YAP2 corresponds to the only WW domain of YAP1, the binding ability of YAP1 to Lats1 and Lats2 was abrogated when its WW domain was mutated (data not shown). In sum, the results presented in Fig. 1, D and E, suggest that the binding between YAP2 and Lats1 (Lats2) is mediated by the WW domains of YAP2 and the PPXY motifs of Lats1 (Lats2).

Lats1 and Lats2 Cooperate with Mst2 to Phosphorylate YAP—Because Drosophila Wts interacts with and phosphorylates Yki, we decided to investigate whether Lats1 and Lats2 phosphorylate YAP in mammalian cells. We employed a gel shift assay, to detect YAP phosphorylation. (For the analysis of phosphorylation sites we used YAP1 because of the battery of mutants that were available in our laboratory.) The band of YAP1 was shifted upward when Mst2 and Lats1 were transfected together (Fig. 2A, upper panel, compare third and first lanes). This mobility shift was abrogated by phosphatase treatment (Fig. 2A, fourth lane), which suggests that this shift is due to protein phosphorylation. A small difference between the first and second lanes (Fig. 2A) in terms of shift of YAP1 implies that there are endogenous kinases that phosphorylate YAP1 in HEK293 cells.

To confirm that Lats1 is the genuine kinase that phosphorylates YAP1, YAP1 together with various combinations of Mst2, Lats1, and Lats2 was transfected into HEK293 cells. The cell lysates were resolved by SDS-PAGE followed by probing with YAP antibody (Fig. 2C, upper panel). YAP1 and vector alone were co-transfected and used as a control (Fig. 2C, upper panel, first lane). No shift was detected in the presence of Lats1 alone or Mst2 alone (Fig. 2C, upper panel, second and third lanes), yet when both Lats1 and Mst2 were present, the band of YAP1 shifted slightly upward (Fig. 2C, upper panel, fourth lane) suggesting that YAP1 is phosphorylated when both Lats1 and Mst2 are present. Mst2 kinase-dead mutant (D164A) could not shift the band of YAP1 when co-transfected with Lats1 (Fig. 2C, upper panel, fifth lane). This result confirms a previous observation showing that the kinase activity of Lats1 is enhanced when Mst2 phosphorylates Lats1 (44). The amino-terminal portion of Lats1 (amino acids 1–587), which lacks the kinase catalytic region but still contains two PPXY sequences, was also not able to shift the band of YAP1 (Fig. 2C, upper panel, sixth lane). This finding further suggested that the kinase activity of Lats1 is indispensable for shifting the protein band of YAP1 upward, implying phosphorylation of YAP1. Lats2 exhibits activity similar to that of Lats1, as Lats2 can be substituted for Lats1 (Fig. 2C, upper panel, seventh lane). These results suggest that Mst2 kinase activates Lats1 or Lats2 kinases, which in turn phosphorylate YAP1.

Ser-127 in YAP1 Is the Target Site Phosphorylated by Lats1—YAP1 is likely to be phosphorylated by activated Lats1 and Lats2. We evaluated the potential phosphorylation sites of YAP1 with the help of the Netphos 2.0 program (Technical University of Denmark). Ten candidate sites of phosphorylation by serine kinases were identified within the YAP1 sequence based on a relative score given by the program. In addition, we included Ser-127, a site that was previously shown to be a target of Akt kinase (45), and Thr-110, which together with Ser-109, were reported as phosphorylation sites of YAP isolated from the nuclei of HeLa cells (46). These 12 sites were individually mutated to alanine (Fig. 2B). Each of the 12 YAP1 mutants was transfected into HEK293 cells together with either control vector or FLAG-Lats1 and FLAG-Mst2. Shift assay was performed as shown in Fig. 2A. With the exception of S127A, every mutant was still phosphorylated by Lats1 and Mst2 (data not shown).

We investigated whether Ser-127 was phosphorylated (Fig. 2C, middle panel) and found that strong signals were detected when YAP1 was transfected with Mst2 and Lats1 (Lats2). These data correlated with the results of the mobility shift assay. Furthermore, the protein band of YAP1 S127A mutant no longer shifted upward even though it was co-transfected with FLAG-Lats1 and FLAG-Mst2 (Fig. 2D). This suggests that Ser-127 is a common phosphorylation site for Lats1 and Akt kinases. In Fig. 2, we used YAP1 to conduct a gel shift assay. The YAP2 protein also has a Ser-127 site, and the protein band was shifted upward as YAP1 was in the presence of Lats1 and Mst2 (data not shown). In our experimental setting, we could not confirm Ser-109 and Thr-
110 of YAP1 as sites phosphorylated by Lats1 kinase. Recent in vitro studies have shown that Lats1 kinase can phosphorylate YAP on several serine residues in addition to Ser-127 (47).

Expression of YAP2 Results in Serum-dependent Detachment of Cells—To study the biological function of YAP, HEK293 cells individually expressing YAP1 WT, YAP2 WT, YAP2 1&2 WW*, YAP2 S127A, and control vector were established in an inducible system. The induction of these proteins had no influence on cell detachment when cells were maintained in DMEM containing 10% FCS. However, when the concentration of serum was reduced to 1%, the effect of YAP2 overexpression on cell detachment was detectable. The induction of YAP2 WT expression in HEK293 cells cultured in DMEM containing 1% FCS caused significant detachment of the cells from the plate compared with controls in which expression of YAP2 was not induced. The number of attached cells was counted following three washes to remove floating cells. The ratio of attached cells with induced YAP2 WT to attached cells without induced YAP2 WT was 0.661 (Fig. 3A, second bar), i.e. the number of attached cells decreased by 33.9% in YAP2 induced cultures. A relatively modest decrease (15.9%) was observed when the double mutant of YAP2 1&2 WW* was induced (Fig. 3A, third bar), suggesting that the WW domains of YAP2 play a key role in reducing the number of attached cells. Interestingly, the effect of YAP2 S127A mutant overexpression on cell detachment was more dramatic than that of YAP2 WT (Fig. 3A, fourth bar). As this mutant is known to preferentially localize in the nucleus (29, 45), it is likely that the function of YAP2 in regulating cell attachment is dependent on its nuclear targets.

We noted that the overexpression of YAP1 was not as efficient as that of YAP2 in terms of reducing cell number (Fig. 3A, fifth bar). The presence of an additional WW domain in YAP2 may account for the observed difference in the cell detachment "readout."

To study the biological function of YAP under more physiological conditions, we reduced the level of endogenous YAP in HEK293 cells by RNAi and measured the rate of cell growth
Interestingly, the cells in which the level of YAP was reduced grew significantly faster compared with the control cells (31- versus 21-fold increase after 5 days of culture). This result suggests that the relative level of YAP in HEK293 cells can regulate the balance between cell growth and cell detachment (apoptosis).

Effect of YAP2 on Reduced Attachment of Cells Is Rescued by Mst2 and Lats1—Because YAP2 S127A mutant had a more significant influence on the number of detached cells than YAP2 WT (Fig. 3A), we postulated that Lats1, Lats2, and Mst2 could regulate this process by phosphorylating YAP at Ser-127. To investigate the effects of Lats1, Lats2, and Mst2 on cell attachment, these proteins were transiently expressed as FLAG-tagged proteins in HEK293 cells that expressed an inducible YAP2 WT. After the transfection of the upstream kinases Lats1, Lats2, and Mst2, we induced expression of YAP2 WT. The number of cells decreased by 28.7% when the expression of YAP2 WT was induced (Fig. 3C, first bar). The effect of YAP2 was partially diminished by Lats1 or Mst2 (Fig. 3C, second and third bars, respectively) and was completely abolished by Mst2 and Lats1 co-transfected prior to the induction of YAP2 WT (Fig. 3C, fourth bar). Lats2 fully replaced Lats1 in this assay (Fig. 3C, seventh bar), suggesting that Lats2 has the same function as Lats1. The role of Lats1 and Mst2 kinases as upstream factors of YAP was further confirmed by the observation that both the Mst2 kinase-dead mutant (D164A) and the Lats1 amino-terminal portion (which lacks kinase activity) were barely able to diminish YAP2 function (Fig. 3C, fifth and sixth bars, respectively). The data presented in Figs. 2 and 3 suggest that upon activation by Mst2 kinase, Lats1 and Lats2 kinases phosphorylate YAP at Ser-127 and inactivate its function as a stimulator of cell detachment.
Expression of YAP2 in HEK293 Cells Promotes Apoptosis—As shown in the cell counting assay described in Fig. 3, when we observed YAP2 WT induced in HEK293 cells, the 96-h time point exhibited the most significant change in the ratio of attached versus floating cells. However, at the 48-h time point the number of floating cells was minimal. Therefore, the cells began to detach from the plate during the 48–96-h time period. Knowing that YAP has been implicated in the apoptotic pathway by stabilizing p73 (35–37), we decided to examine whether apoptosis could be detected in cells overexpressing YAP2. We assayed the total population of cells that included both the attached cells and floating cells. It is known that the 113-kDa poly(ADP-ribose) polymerase (PARP) is cleaved during apoptosis into 89- and 24-kDa fragments, serving as a marker of cell death. In the absence of YAP2 WT, the cleavage fragment of PARP was barely detectable (Fig. 4A, upper panel, third lane). However, in the presence of YAP2 WT, the 89-kDa cleavage fragment was clearly detectable (Fig. 4A, upper panel, fourth lane), suggesting that expression of YAP2 promotes apoptosis in HEK293 cells. YAP2 S127A was also capable of generating the cleaved fragment of PARP (Fig. 4A, upper panel, eighth lane). However, when the WW domains of YAP2 were mutated, the cleaved band of PARP was barely detectable (Fig. 4A, upper panel, sixth lane). This result suggests that WW domains of YAP2 play a crucial role in promoting apoptosis. Interestingly, YAP1 failed to generate a cleaved fragment of PARP (Fig. 4A, upper panel, 10th lane).

To rule out the possibility that the YAP-induced apoptosis is an HEK293 cell line-specific phenomenon, we used another cell line, NIH3T3, in which we expressed YAP1 WT, YAP2 WT, YAP2 1&2 WW*, and YAP2 S127A mutant and control vector, each in an inducible fashion. The cell counting assay and the apoptotic assay with PARP antibodies were performed in the same manner as for HEK293 cells. The data showed that NIH3T3 cells behaved similarly to HEK293 (Fig. 4B), although the observed effects of YAP2 WT expression in NIH3T3 cells were not as strong as those in HEK293 cells. The proapoptotic tendency of cells with overexpressed YAP was common in both cells. The fact that YAP2 S127A, a mutant that localizes in the nucleus, was a strong proapoptotic factor in both cells suggests that the nuclear localization of YAP2 is crucial for its proapoptotic function.

To determine when the apoptotic markers are detected after the induction of YAP2 WT, we analyzed the time course of its effects in HEK293 cells (Fig. 4C). There was almost no appearance of PARP or active caspase-3 at the 24-h time point after the YAP2 WT induction. However, at the 48-h time point, a weak signal of PARP cleavage was detected, and active caspase-3 was clearly detected in cell lysates with YAP2 WT. At that point we did not observe cell detachment. PARP cleavage was clearly detectable, and cell detachment began to occur at 72 h and became robust at the 96-h time point.

The up-regulation of cyclin E was shown previously as a downstream effect of YAP overexpression, which leads to growth promotion and neoplastic transformation (12, 31). Therefore, we decided to determine the levels of cyclin E in our system. In contrast to previous observations, YAP2 WT overexpression did not up-regulate cyclin E in HEK293 cells (Fig. 4C).

Because the results shown in Fig. 4A demonstrate a correlation with the data presented in Fig. 3A, we considered that the cell counting assay might measure the degree of apoptosis in HEK293 cells. YAP2 WT and YAP2 S127A are competent in terms of promoting apoptosis, whereas YAP2 1&2 WW* and YAP1 lack this ability. Next, we investigated the effects of Lats1 and Mst2 on PARP cleavage, because the effect of YAP2 WT on reduced attachment of cells was rescued by Lats1 and Mst2 (Fig. 3C). As anticipated, the ability of YAP2 WT to promote PARP cleavage was completely abrogated when both Lats1 and Mst2 were present (Fig. 4D, upper panel, compare fourth and second lanes).

Both WW Domains in YAP2 Are Required to Bind to p73—Our data indicate that YAP2 promotes apoptosis of HEK293 cells in a serum-dependent fashion. This proapoptotic function was decreased when both Lats1 and Mst2 were co-expressed with YAP2 and phosphorylated at Ser-127.

To elucidate the downstream signaling factors of YAP, which are implicated in apoptotic function, we focused on p73, a member of the p53 family. It was shown that YAP interacts functionally with p73 and increases the stability and accumulation of p73 in response to DNA damage (35–37). However, the previous study did not determine whether the WW domains of YAP2 played a role in p73 stabilization. YAP1 WT and YAP2 WT, as well as S127A, S127D and WW domain mutants, were transiently expressed in HEK293 cells together with HA-p73. The cell lysates were immunoprecipitated with FLAG antibody followed by immunoblotting using HA antibody (Fig. 5A). Only YAP2 WT, YAP2 S127A, and YAP2 S127D formed a complex with p73, whereas all other YAP WW domain mutants failed to precipitate HA-p73. Surprisingly, YAP1 WT also failed to precipitate p73. This result indicates that YAP2, with intact WW domains, is a cognate partner of p73.

Functional WW Domains of YAP2 Are Required for Stabilization of p73—The fact that YAP2 with intact WW domains could bind p73 prompted us to further study the effects of YAP2 on p73. As p73 was shown to be stabilized by YAP in response to DNA damage (35–37), we decided to investigate whether stabilization of p73 by YAP2 takes place in our own cellular system. As before, we challenged HEK293 cells with low serum (1%). YAP2 WT was expressed as an inducible protein, and cells were transfected with HA-p73. Expression of YAP2 was induced, and the levels of HA-p73 were monitored as a function of time (Fig. 5B, upper panel). After 72 h, the expression of HA-p73 showed a 2.21-fold difference between YAP2-induced and noninduced cells (Fig. 5B, upper panel, compare sixth lane with fifth lane). This difference became larger (2.75-fold) at the 96-h time point (Fig. 5A, upper panel, compare eighth lane with seventh lane). In the absence of YAP2, the expression level of HA-p73 decreased, suggesting a stabilizing effect of YAP2 on p73.

As we already knew that the WW domains of YAP2 are indispensable in promoting apoptosis (Fig. 4A) and binding to p73 (Fig. 5A), we looked into the role of WW domains to stabilize p73. The induced expression of YAP at 96 h represented the ideal time point to visualize stabilization of p73 by...
FIGURE 4. YAP2 promotes apoptosis.

A, YAP2 WT and YAP2 S127A generate a cleaved fragment of PARP. The expressions of control vector, YAP2 WT, YAP2 1&2 WW*, YAP2 S127A, and YAP1 WT were individually induced in HEK293 cells and maintained in DMEM containing 1% FBS for 96 h. Crude cell extracts were resolved on SDS-PAGE and immunoblotted (IB) with PARP antibody (upper panel), YAP antibody (middle panel), and GAPDH antibody (lower panel). B, induction of YAP2 expression results in reduced cell attachment in NIH3T3 cells. The expressions of each construct, control vector, YAP2 WT, YAP2 1&2 WW*, YAP2 S127A, and YAP1 WT, were individually induced in NIH3T3 cells maintained in DMEM containing 2.5% FBS for 96 h. After the removal of detached cells, attached cells were trypsinized and their numbers counted. The ratios of number of induced cells to number of noninduced cells for each induced protein are shown. The lower panels show Western blots that verify the expression of the indicated proteins. C, time course of the effects of YAP2 WT induction in HEK293 cells. The expression of YAP2 WT was induced in HEK293 cells and maintained in DMEM containing 1% FBS for the indicated periods of time. After the removal of detached cells, attached cells were trypsinized and their numbers counted. The ratios of the number of induced cells to number of noninduced cells are shown. The lower panels show immunoblots verifying the expression of the indicated proteins. D, the combination of Lats1 and Mst2 spoils the ability of YAP2 to generate cleaved fragment of PARP. Either FLAG control vector or FLAG-Lats1 with Flag-Mst2 were transfected into HEK293 cells capable of expressing YAP2 WT in an inducible system. 24 h after transfection, the cells were distributed into new plates, and the expression of YAP2 WT was induced by tetracycline. 96 h post-induction, cells were trypsinized and analyzed as described in A. The cleaved fragment of PARP was monitored (upper panel). The expression of induced YAP2 WT, transfected FLAG-tagged proteins, and GAPDH was also checked by immunoblotting.
YAP2 WT (Fig. 5B). As we expected, the amount of p73 was increased when YAP2 WT or YAP2 S127A were induced, whereas YAP2 1&2 WW* and YAP1 WT failed to stabilize p73 (Fig. 5C). As shown previously (37), we also used cycloheximide treatment of cells to enhance the detection of protein stability and showed that the amount of HA-p73 clearly increased in the presence of YAP2 or YAP2 S127A (Fig. 5D). These data further support our results (Fig. 5C).

To investigate whether p73 is the major molecule to take over the proapoptosis function of YAP2, we conducted RNAi treatment in HEK293 cells and evaluated its effect on cell detachment in the presence of YAP2 WT. After the p73-directed RNAi treatment, the expression of endogenous p73 was completely abrogated (Fig. 5E). The p73-specific RNAi oligo or control RNAi oligo was transfected into HEK293 cells followed by the induction of YAP2 WT expression. After 96 h of culture in DMEM containing 1% FCS, the endogenous p73 was not detectable in either case (data not shown). This could possibly be due to the short half-life of endogenous p73. The cells were counted as described for Fig. 3. When control RNAi was transfected, the ratio of attached cells with induced YAP2 WT to attached cells without induced YAP2 WT was 0.525 (Fig. 5F). However, when p73 RNAi was transfected, the ratio was increased to 0.735 (Fig. 5F), indicating that the proapoptotic function of YAP2 was partially impaired when p73 was removed. This result supports the role of YAP2 in promoting apoptosis via p73. In sum, YAP2 binds to p73 via WW domains, leading to the stabilization of p73 and promotion of apoptosis.

Lin1 Controls the Localization of YAP2 in HEK293 Cells—YAP is a transcriptional co-activator, and its protein product is detected both in the cytoplasm and the nucleus (29). The WW domain of YAP interacts with several transcription factors, and its localization in the nucleus correlates with its co-activating role in transcription (30, 48, 49). The S127A YAP mutant was shown to enter the nucleus and stimulate transcription more efficiently than the WT YAP (29). Because Lats1 forms a complex with YAP through a PPPY

FIGURE 5. YAP2 is able to stabilize p73. A, both WW domains of YAP2 are required to bind to p73. HA-p73 with the indicated YAPs was transfected into HEK293 cells. Lysates were immunoprecipitated (IP) with FLAG antibodies, resolved on SDS-PAGE, and immunoblotted (IB) with HA antibodies (upper panel). The expression levels of total HA-p73 (middle panel) and FLAG-tagged proteins (lower panel) were also monitored. B, time course of degradation of HA-p73 in the presence of YAP2. HEK293 cells that express YAP2 WT in an inducible system were transfected with HA-p73. 24 h later, the cells were plated in fresh DMEM containing 1% FBS, blasticidin (5 μg/ml), and zeocin (200 μg/ml). Tetracycline (1 μg/ml) was added to the medium to induce the expression of YAP2 WT. At 72 h and 96 h after induction, the cells were harvested followed by immunoblotting using antibodies of HA (upper panel), YAP (middle panel), or GAPDH (lower panel). C, the WW domains of YAP2 are required to stabilize the expression of p73. HEK293 cells that express control vector, YAP2 WT, YAP2 1&2 WW*, YAP2 S127A, or YAP1 WT in an inducible system were transfected with HA-p73 followed by analysis as described in B. Tetracycline was added to the medium for 96 h. D, YAP2 stabilizes p73. HA-p73 with the indicated YAPs was transfected into HEK293 cells, and each plate was divided into two. 24 h later, one plate was harvested, and 300 μg/ml of cycloheximide (CHX; Sigma) was added to the other plate and harvested after additional 24 h. Cell lysates were immunoblotted using antibodies to HA (upper panel), YAP (middle panel), or GAPDH (lower panel). E, endogenous p73 was removed by p73-specific RNAi. Control RNAi oligo or p73-specific RNAi oligo was transfected to HEK293 cells that express YAP2 WT in an inducible system, and the expression of endogenous p73 was monitored by p73 antibody. F, removal of endogenous p73 weakened proapoptotic function of YAP2. 48 h after the transfection of RNAi oligos in E, cells were scattered into fresh plates and maintained in DMEM containing 1% FCS. Expression of YAP2 WT was induced by adding tetracycline. 96 h later, the numbers of cells were counted, and expression of YAP2 WT was monitored as described in the legend for Fig. 3.
sequence-WW domain association, we suspected that the localization of YAP is affected by Lats1.

To characterize human orthologs of the Drosophila Hpo pathway in terms of protein localization, we expressed the individual proteins WW45, Mst2, Lats1, and YAP2 as GFP-tagged products in HEK293 cells and determined the locale of each protein by fluorescence microscopy (Fig. 6A). WW45, Mst2, and Lats1 localized in the cytosol. However, GFP-YAP2 was detected in both the nucleus and cytosol. We divided cells that express GFP-YAP2 into two categories: “localized in the cytosol” and “localized in the nucleus.” 65.3% of the cells that expressed GFP-YAP2 localized in the cytosol, whereas 34.7% of them localized in the nucleus. Representative images of protein localizations are shown in Fig. 6A.

To know whether Lats1 affects the localization of YAP2 in cells, pDsRed-YAP2 WT or pDsRed-YAP2 S127A was co-transfected with GFP-Lats1 WT or GFP-Lats1 PY1* & 2* (Fig. 6B). Using fluorescence microscopy, the localizations of pDsRed-YAP2 WT, pDsRed-YAP2 S127A (Fig. 6B, upper row), and GFP-tagged proteins (Fig. 6B, lower row) were monitored. In the presence of GFP-Lats1 WT, the pDsRed-YAP2 WT seldom localized in the nucleus, and the silhouette of the nucleus was clearly discernible when pDsRed-YAP2 was observed in cells. The percentage of cells in which pDsRed-YAP2 WT was observed in the nucleus was only 10.7% (Fig. 6, B and C). However, when GFP-Lats1 PY1* & 2* was present, the localization of pDsRed-YAP2 WT changed. The percentage of pDsRed-YAP2 WT localized in the nucleus increased significantly to 35.3% (Fig. 6, B and C). A similar tendency was observed when YAP2 S127A was expressed instead of YAP2 WT. The YAP2 S127A protein is likely to localize in the nucleus; 38.3% of YAP2 S127A localized in the nucleus in the presence of GFP-Lats1 WT. However, this percentage dramatically increased to 83.0% in the presence of GFP-Lats1 PY1* & 2*, a mutant that lacks the ability to associate with YAP2.

These results suggest that Lats1 prevents YAP2 from entering the nucleus and that the binding ability of Lats1 to YAP2 controls the localization of YAP2. The regulation of YAP localization is critical for its function; the most recent report implicates YAP in the control of cell contact inhibition (34). This activity of YAP correlated well with the subcellular localization of YAP.

**DISCUSSION**

The Hpo pathway controls the intrinsic size of organs by coordinating proliferative and apoptotic signals. Thus the elucidation of the molecular mechanism of this pathway should help us in understanding the process of normal development and its numerous pathological aberrations, including cancer.

We reconstituted the core of the Drosophila Hpo pathway in human epithelial kidney cells, focusing on the role of WW domain-mediated complexes, which are particularly prevalent in this pathway. By expressing tagged human orthologs of the Hpo pathway in HEK293 cells, we documented that Mst2 and Lats1/2 kinases act upstream of YAP1/2 transcriptional co-activators. These two kinases cooperate in phosphorylating YAPs (YAP1 and YAP2) at the Ser-127 site. Akt kinase is known to induce accumulation of YAP in the cytosol rather than in the nucleus by phosphorylating YAP at Ser-127 (45). Our results show that Lats1 by itself can anchor YAP2 in the cytosol, and the direct binding between PPXY motifs of Lats1 and WW domains of YAP2 controls the localization of YAP2. Lats1 retains YAP2 in the cytosol in two different ways: by phosphorylating YAP at Ser-127 and creating the 14-3-3 anchor site and by forming a direct protein-protein complex. The YAPs that evade contacts with Lats1 enter the nucleus freely and promote apoptosis of cells by stabilizing p73, a proapoptotic member of the p53 family (Fig. 7).

The following aspects of this study deserve further comment: (i) the apparent paradox of YAP signaling as an oncopgene or promoter of apoptosis; (ii) the role of the WW domain in the specificity of signaling complexes of the Hpo pathway; (iii) the modulating function of isoforms of Mst, Lats, and YAP in the Hpo pathway output; (iv) the differentiation between apoptosis versus anolikis in the cell assay; and (v) the usefulness of the apoptotic readout in HEK293 cells for the identification of
the upstream factors that initiate the cascade of Hpo pathway, ultimately controlling the size of organs.

Recent reports have suggested that human YAP is a candidate oncogene because it is a part of the 11q22 amplicon detected in several cancer types (31, 32). Gene expression studies have shown that in liver cancer, YAP is co-amplified with a gene that encodes an inhibitor of apoptosis, cIAP1, and both contribute to the development of hepatocellular carcinoma (31). In MCF-10A mammary epithelial cells, the overexpression of YAP was shown to cause oncogenic transformation (32). These two observations seem to contradict previous reports of YAP acting as an activator of apoptosis in response to DNA damage (35–37). However, we do not consider these reports contradictory. The “YAP paradox” is consistent with the still emerging, yet widely accepted notion that signaling proteins often play diverse roles depending on the cellular context, i.e. the repertoire of signaling molecules available in given cells or tissues. The comparison of signaling proteins with electronic components in which the effects depend on their specific placement within the entire electrical circuit is an accepted analogy in the biology of signal transduction systems (50). Therefore, YAP should be considered a facultative oncogene. In liver cells, but not in other cells of the body that contain the same 11q22 amplicon, YAP may cooperate not only with cIAP1 but also with liver-specific genes to cross the threshold required in hepatocytes for oncogenesis. Similarly, the overexpression of YAP in mammary epithelial cells elicits transformation, perhaps because YAP collaborates in these cells with the amplified myc (51). Simple overexpression of YAP in certain epithelial or fibroblastic cells (HEK293, NIH3T3) does not cause oncogenic transformation, unless additional genes are co-expressed or amplified (32, 52). The presence of overexpressed c-myc in systems where YAP caused transformation suggested that for YAP to be oncogenic requires the expression of an additional gene that encodes the anti-apoptotic protein (such as c-Myc, cyclin E, or Runx2). Our work supports a model of functional dichotomy of YAP. If the interaction partners of YAP (such as c-myc or cyclin E) in given cells or organs are anti-apoptotic, then YAP is oncogenic. If partners of YAP are proapoptotic, such as p73, and cyclin E is not induced by YAP, then YAP is proapoptotic. In HEK293 and NIH3T3 cells, YAP interacts with p73 and does not induce cyclin E. Because Drosophila does not have the p73 homolog, Yorkie (dYAP) may be unable to engage in proapoptotic signaling directly. Moreover, cell-specific and varying levels of endogenously expressed p63 and p73 (members of the p53 family that interact with YAP) may tip the balance from transformation to apoptosis in mammalian cells (53). The tissue-specific expression of YAP targets, namely the members of the TEAD/TEF family of transcription factors, also could be responsible for the dichotomy of YAP/Yki function as oncogenic or proapoptotic factor (54–57). Intuitively, it seems that a protein that has the capability to sense the signaling background of cells and mediate appropriately such diverse processes as proliferation or apoptosis would be ideal for controlling the size of organs and organisms during development and beyond. YAP and Yki are nuclear effectors of the Hpo pathway acting as rheostats for proliferation and apoptosis.

The WW domain was mapped in the human proteome using a domain-peptide interaction screen (58). By using more than 69,000 data points and assigning relative strength of binding between 57 WW domains and 1830 proline-rich peptides corresponding to known proteins, several new complexes and strings of interactors emerged. Yet none of the map-predicted pathways or currently known signaling pathways is as “rich” in the WW domain-PPXY/PPXF complexes as the Hpo pathway. All components of the core of the Hpo pathway contain either WW domains or their cognate ligand motifs, PPXY or PPXF (Figs. 1A and 7). Two aspects of the WW domain should be considered in future studies of the pathway. (i) Because the WW domain complexes can be inhibited with small non-peptide or peptoid compounds, the use of such inhibitors in the biochemical analysis of the pathway should be illuminating.
The reason for multiple isoforms of the proteins Mst, Lats, and YAP in the human pathway, compared with the single orthologs in the Drosophila pathway, is not clear. Our data indicate that YAP2 seems to be a more potent regulator of apoptosis than YAP1. Most recently, YAP1 (but not YAP2) was shown to be expressed in stem cells and was implicated in the expansion of undifferentiated progenitor cells (61). Perhaps other differences apply to isoforms of Mst and Lats kinases. Small changes in the amino acid sequence among the isoforms may translate into important changes in their activities. Alternatively, the isoforms represent tissue-specific variants that are expressed mostly as single proteins in a given tissue or organ. Additional work needs to be done on the tissue-specific expression of the mammalian orthologs of the Hpo pathway in order to understand the potential regulatory function of the isoforms on the output of the pathway. It should be noted that more isoforms of the Hpo pathway could be identified in the fly and in human proteins in the near future. We are aware of the isoform of Yki, which contains one WW domain and could be considered a YAP1 ortholog. In mammals, there are additional isoforms of YAP, which either contain small insertions (29) or large deletions of the transcriptional activation domain and are involved in controlling cell death in neurons (49).

Because in our biological readout we observed both cell detachment and apoptosis, we needed to consider the possibility that what we observed was a specific kind of cell death, known as anoikis. Anoikis is a program of detachment-induced cell death. To differentiate between anoikis and apoptosis, we separated the attached cells from the floating cells after 96 h of YAP2 overexpression and probed for PARP cleavage products. We found a similar level of PARP cleavage in attached and in floating cells (see supplemental Fig. 1). Actin ccapase-3 was detected in both the attached cells and the floating cells when the expression of YAP2 WT was induced, whereas it was not detected in the control, noninduced cells. These results strongly suggest that YAP2 causes true apoptosis and not anoikis. We also preformed a DNA ladder assay on the YAP2-overexpressing cells and confirmed the apoptosis by an alternative biochemical test (data not shown).

The apoptotic readout of the Hpo pathway in HEK293 cells described here represents a useful experimental system for the identification of the putative upstream receptor or membrane protein that initiates an entire signaling cascade and ultimately controls the size of organs. Because HEK293 cells are easy to maintain and the efficiency of DNA transfection in these cells is high, one could design a simple screen for genes that encode membrane proteins and subsequently affect the apoptotic response significantly. Considering the high level of conservation among the known orthologs that constitute the pathway, priority in such a screen should be given to those genes that encode membrane proteins and are well conserved between flies and mammals (62–67).
