TUMOR SUPPRESSOR LATS1 IS A NEGATIVE REGULATOR OF ONCOGENE YAP

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Running title: phosphorylation and regulation of YAP by LATS1

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LATS (Large Tumor suppressor) or warts is a Serine/Threonine (Ser/Thr) kinase that belongs to Ndr/LATS subfamily of AGC [protein kinase A (PKA)/PKC/PKC] kinases. It is a tumor suppressor gene originally isolated from Drosophila and recently isolated from mice and humans. Drosophila or mice mutant for LATS develop tumors in various tissues. Recent studies in Drosophila demonstrate that LATS is a central player of an emerging tumor suppressor pathway called Hippo-LATS/Warts pathway that suppresses tumor growth by regulating cell proliferation, cell growth, and cell death. Although tremendous progress has been made toward our understanding the roles of LATS in tumorigenesis, the kinase substrates of LATS or downstream target proteins mediating LATS function remain largely unknown. In this study, we have provided convincing evidence that LATS can bind to and phosphorylate transcription regulator and oncogene YAP in vitro and in vivo. We have also identified HXR/H/KXXXS/T as the consensus phosphorylation sequence for LATS/Ndr kinase substrates. Significantly, we have discovered that LATS inactivates YAP oncogenic function by suppressing its transcription regulation of cellular genes via sequestration of YAP in the cytoplasm after phosphorylation of YAP. Finally, by using microarray analysis, we have also identified many oncogenes or tumor suppressor genes up-regulated or down-regulated by YAP. These research findings will have profound impacts on our understanding the molecular mechanism of the LATS tumor suppressor and the emerging Hippo-LATS/Warts pathway.
Ndr kinase members include budding yeast dbf2/20 and cbkl, fission yeast orb6 and sid2, C. elegans sax-1, Drosophila trc and LATS/warts and mammalian LATS1, LATS2, Ndr1 and Ndr2 kinases, which are involved in the regulation of morphogenesis, cell division, cell survival, centrosome duplication, and neural outgrowth and dendrite tiling (27). Although it has been shown that the kinase activity is essential for the functions of these Ndr family kinases (6-7, 28-31), their downstream kinase substrates are still largely unknown. Identification of these substrates and characterization of their interactions with Ndr kinases will be crucial to elucidate the molecular mechanism of their functions. Recently, Yokie was identified as a novel kinase substrate and downstream target of Drosophila LATS. Drosophila LATS can suppress cell proliferation and cell growth by phosphorylating and inhibiting Yokie function (15). However, the molecular mechanism by which LATS phosphorylates and inactivates Yokie remains unknown. Recently, the human homolog of Yokie, YAP, was identified as an oncogene in breast and liver tumorigenesis (25-26). Overexpression of YAP induced transformation of mammalian cells and tumor formation in mice (25-26). However, whether and how human LATS1 and LATS2 phosphorylate and regulate YAP function remains to be determined. In addition, although YAP was identified as transcription co-activator and oncogene in mammalian cells, the downstream genes regulated by YAP during YAP-induced transformation have not been identified.

In this study, we have discovered that LATS1 can interact with and phosphorylate YAP both in vitro and in vivo. Significantly, we have provided convincing evidence that LATS1 can negatively regulate transcription regulation and transformation functions of YAP by inhibiting its nuclear translocation via phosphorylation of multiple sites in YAP. By using peptide kinase assays, we have also identified the consensus phosphorylation sequence for LATS1 and Ndr1 kinase substrates. Our findings clearly indicate that LATS/Ndr has similar substrate specificity to those of other AGC kinase members.

**EXPERIMENTAL PROCEDURES**

Plasmid construction and site-directed mutagenesis—Full length cDNAs of human LATS1 (Accession No. NM_004690), LATS2 (Accession No. NM_014572), YAP (BC 038235, IMAGE:5747370) or Mst2 (NM_006281) were subcloned into pcDNA3.1-hygro-3×FLAG, pcDNA3-HA, pcDNA3.1-myc vectors, respectively. For lentivirus production, LATS1 or YAP or Mst2 cDNA was first amplified by polymerase chain reaction (PCR), digested by Pme I and subsequently cloned into PmeI site of WI lentiviral vector. The following primers were used for PCR: LATS1, sense primer 5’-AGCTTTGTTTAAAACCATGGAGCGAAAAAGC CATTTCTGAAGAGGACTTTGAATGTTTTCA TGAAGAGGAGTG-3’ (Pme I site is underlined and Myc tag is boldface) and anti-sense primer 5’-AGCTTTGTTTAAAACCATATACATAG ATC-3’ (Pme I site is underlined); YAP, sense primer 5’-AGCTTTGTTTAAAACCATGGAGGATA CCCATACGCCTCCAGACTCCCGGAT CCCGGGCAGCAAGCG-3’ (Pme I site is underlined and HA tag is boldface) and anti-sense primer 5’-AGCTTTGTTTAAAACCATATACATAG ATC-3’ (Pme I site is underlined); Mst2, sense primer 5’-AGCTTTGTTTAAAACCATATACATAG ATC-3’ (Pme I site is underlined and HA tag is boldface) and anti-sense primer 5’-AGCTTTGTTTAAAACCATATACATAG ATC-3’ (Pme I site is underlined).

Cell culture, transfection, and immunofluorescence—Cell culture, cell transfection and immunofluorescence staining are described (8). Images were obtained with Nikon Eclipse TE-2000U Inverted Fluorescent Microscope (Nikon).

Peptides, antibodies, co-immunoprecipitation (Co-IP), protein fractionation and western blot—All the peptides used in this study were synthesized by GL Biochem (Shanghai, China). Mouse monoclonal antibodies to Myc (Roche), FLAG (Sigma), α-tubulin (Sigma), and PCNA (Santa Cruz) were 9E10, M2, B-5-1-2, and F2, respectively. Rabbit polyclonal antibodies to HA (Y11) and YAP (H125) were purchased from Santa Cruz. Rabbit α-LATS2 polyclonal antibody (BL2213) was purchased from Benthyl.
Laboratories, Inc. Mammalian cell protein extraction, cytoplasmic and nuclear protein fractionation, and Co-IP were as described (8, 32).

**Fusion protein production and GST-pull down assay**—GST fusion proteins were produced and purified as described (8). For GST-pull down assay, about 50-200 μg of protein lysate expressing LATS1/2-FLAG, Ndr1/2-FLAG or YAP-HA were mixed with 10 μg of GST (control) or LATS1 or YAP GST fusion proteins on beads, and incubated at 4°C with rotating for 2 h. The beads were washed 4 times with 1%NP40 lysis buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 mM EDTA, 1.0% NP40, and 1 × proteinase inhibitor), resuspended in 2× sodium dodecyl sulfate (SDS) sample buffer, boiled, centrifuged, and the supernatants were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis using either α-FLAG or α-HA antibody.

**Lentivirus production, purification, titration, and infection**—One day before transfection, 5×10^6 293T cells were plated on a 150-mm plate coated with 0.1 mg/ml poly-L-lysine and incubated at 37°C overnight. Then, 7.5 μg of LATS1-Myc-WPI or YAP-HA-WPI or Mst2-FLAG-WPI transfer vector were mixed with 5.6 μg of PAX (packing) and 1.9 μg of MD2G (envelop) plasmids and 37.5 μl of LipofectAMIN2000. After incubation for 20 min at room temperature, the mixture was added dropwise into each plate. The medium was replaced with 18 ml of OPTI-MEM I medium (serum free) containing 10 mM Na Butyrate (demethylation of plasmids to increase gene expression) 20-24 h after transfection. Two days after transfection, the media containing lentivirus were collected, passed through a 0.45 μm filter and concentrated using Centricon-20 ultrafiltration column (Millipore). For virus titration, 4×10^4 HeLa cells were plated into each well of a 24-well plate containing coverslip coated with fibronectin. The cells were subject to immunostaining using rabbit α-YAP polyclonal antibody after cultured at 37°C overnight.

**RNA interference knockdown of LATS**—A TriFECTa kit containing a Dicer-substrate (ds) siRNA duplex targeting human LATS1 or LATS2 mRNA and a negative control siRNA with scrambled sequence absence in human genome were purchased from Integrated DNA Technologies (Coralville, USA). Transfection of HeLa cells with 10 nM siRNA was performed using Lipofectamine2000 (Invitrogen) according to manufacturer’s instructions. Four days post-transfection, protein was extracted and knockdown of LATS1 and LATS2 was confirmed by western blot. For immunostaining of YAP after LATS knockdown, HeLa cells were re-plated into a well of 24-well plate containing coverslip coated with fibronectin. The cells were subject to immunostaining using rabbit α-YAP polyclonal antibody after cultured at 37°C overnight.

**RNA isolation, microarray analysis and real-time quantitative reverse transcription (qRT)-PCR**—MCF10A cells grown in 6-well plate were infected with lentivirus expressing vector (control) or YAP-HA with MOI of 2. Duplicate MCF10A cells in different wells of 6-well plate (biological replicate) were infected with YAP lentivirus. Two days after infection, total RNAs were extracted with TRIzol reagent (Invitrogen) according to standard protocol. To clear up residual genomic DNA, total RNA is further cleaned using an RNeasy Mini Kit (Qiagen). The quality of RNA is examined by electrophoresis on 1.5% agarose gel and Agilent 2100 Bioanalyzer (version B/02.02) analysis.

For microarray analysis, 250 ng of total RNA were labeled with either Cy3-CTP or Cy5-CTP (PerkinElmer) and subsequently amplified with Low RNA Input Linear Amplification kit (Agilent) according to the instruction from the manufacture. The RNAs were labeled, amplified
and used for microarrays as the following: Array#1: Control/Cy3-CTP, YAP-1/Cy5-CTP; Array#2: Control/Cy3-CTP, YAP-2/Cy5-CTP (biological replicate); Array#3: YAP-1/Cy3-CTP, Control/Cy5-CTP (flip color). About 825 ng of each Cy3 or Cy5 labeled complimentary RNAs (cRNAs) were mixed and hybridized to a 4×44K whole Human Genome Oligo (60mer) Array (Agilent) at 65°C for 17 h in a microarray rotating oven (SciGene). The slides were washed and subsequently scanned on an Agilent Microarray Scanner G2565BA. Scanned images were analyzed with Agilent Feature Extraction Program (version 9.1). YAP up-regulated and down-regulated genes were selected according to the following selection criteria: only those genes whose transcription had changed more than two-fold (LogRatio>=0.3 or <=-0.3) in 2 out of 3 microarrays with a P value of <0.0001 were further evaluated. GeneOntology analysis in GeneTools (http://www.genetools.microarray.ntnu.no) and Pubmed gene search were used to identify the functions of each differentially expressed gene.

SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) was used for real-time qRT-PCR to quantify the level of each differentially expressed gene identified from microarray analysis. In brief, triplicate of 0.2 μg of total RNA extracted from MCF10A cells infected with lentivirus expressing vector (MCF10A-control) or YAP (MCF10A-YAP) were mixed with SuperScript III RT/Platinum, 2× SYBR Green reaction mix, and gene-specific forward and reverse primers in 25 μl. RT-PCR was run at 1 cycle of 50 °C for 5 min, 95 °C for 5 min and 40 cycles of 95 °C for 15 seconds, 60 °C for 30 seconds, and 40 °C for 1 min using an ABI PRISM 7700 Sequence Detection System. 18S rRNA was used as internal control of RNA amounts in each sample. The mRNA level of each gene in MCF10A-control cells relative to that in MCF10A-control was calculated as the following formula: 2^{(ΔΔCt)}(YAPACT-ΔΔCt), where ΔΔCt= cellular gene average Ct-rRNA average Ct. The mean and standard deviation (SD) were calculated from Ct values of triplicate real-time RT-PCR reactions for each RNA sample.

Kinase assays— COS-7 cells transfected with wild-type (WT) or kinase-dead (KD) LATS-FLAG or Ndr-FLAG alone or together with Mst2- myc were lysed in 1%NP40 lysis buffer supplement with 1mM DTT and 1×Phosphatase inhibitor (Phl) (Sigma). For in vitro IP-kinase assay, 100 μg of protein lysate were mixed with 2 μg of α-FLAG (M2) monoclonal antibody together with Protein G beads and incubated at 4 °C for 3 h. The beads were washed twice with 1% NP40 lysis buffer/1 mM DTT, once for 10 min with 1% NP40 lysis buffer/500 mM NaCl/1 mM DTT at 4 °C (rotating) and twice with 20 mM Tris-HCl (pH7.4)/1 mM DTT/1×Phl. The washed beads were then mixed with 2 μg of YAP-GST substrates in a kinase buffer [20 mM Tris-HCl (pH7.5), 5 mM MgCl₂, 5 mM MnCl₂, 1×Phl, 2 mM DTT, 10 μM ATP, 5 μCi γ-ATP³²] and incubated at 30 °C for 30 min. The reaction was stopped by adding 7 μl of 5×SDS sample dye, boiled at 100 °C for 5 min, and subject to SDS-PAGE. After electrophoresis, the proteins were transferred to a nitrocellulose membrane, stained with 1% Ponceau S for visualization of fusion protein substrates, and exposed to autoradiograph film for 0.5-2 h. To test phosphorylation of YAP by LATS1 in vivo, YAP-HA was transfected alone or together with LATS1-FLAG/Mst2-myc into COS-7 cells. The cells were lysed as described above. Ten μg of cell lysate was untreated or treated with 1 U of calf intestinal alkaline Phosphatase (CIP) at 37 °C for 1 h and subject to western blot analysis using α-HA antibody.

For in vitro peptide kinase assays, the precipitated and washed beads were mixed with 3 mM each peptide in a kinase buffer and incubated at 30 °C for 30 min. The reaction mix was spotted on a P81 circle filter paper (Whatman) and washed 4 times with 1% phosphoric acid, dried and subject to CPM (count per minute) radioactivity counting. P81 spotted with reaction mix without peptide was used as background control. The relative kinase activity of specific peptide is calculated as the percentage of CPM for this peptide relative to the CPM for wild-type YAP-S127 peptide.

Protein fractionation and Soft-agar assays— Protein fractionation of cytoplasmic and nuclear proteins and soft-agar assays were as described (8, 32). Colony numbers were counted using the colony count program in Gel Doc EQ system (Bio-rad).
RESULTS

Interaction of LATS1 and YAP in vivo and in vitro—We first used Co-IP assays to examine whether LATS1 interacted with YAP in vivo. FLAG-tagged LATS1 and HA-tagged YAP expression vectors were either transfected alone or together into COS-7 cells. When cell lysates were precipitated with α-HA antibody, LATS1-FLAG was found in the YAP-HA immune complex (Fig. 1A). To eliminate the possibility that LATS1 interacts with YAP is due to their high level of expression in the cells, we also examined the interaction of endogenous LATS1 with YAP in HeLa cervical carcinoma cells expressing high levels of both LATS1 and YAP. Interestingly, endogenous LATS1 was detected in the immune complex precipitated with α-YAP antibody (Fig. 1B).

Next, we used GST-pull down assay to examine whether LATS1 directly interacted with YAP in vitro. As expected, LATS1-FLAG was able to bind strongly to YAP-GST in vitro (Fig.1C). We also examined whether other members of the Ndr kinase family members in mammals, LATS2, Ndr1 and Ndr2, interacted with YAP. Like LATS1, LATS2-FLAG was able to bind to YAP-GST, whereas both Ndr1 and Ndr2 were unable to interact with YAP-GST (Fig. 1C), suggesting that LATS1 and LATS2 are the only two members of Ndr kinase family in mammals that interact with YAP.

Since LATS and Ndr are conserved only in their C-terminal kinase domain, this suggests that LATS may interact with YAP with its N-terminal domain. To confirm this possibility and further understand the molecular mechanism of LATS-YAP interaction, we attempted to map the domains in both LATS and YAP responsible for their interactions. YAP belongs to Group I of the WW domain proteins that bind their partners through their WW domains to PPXY (P, proline; X, any amino acid, Y, tyrosine) or PPRXXP or PPPPP motifs in their binding partner (33). LATS1 contains two PPXY (373PPXY376 and 556PPXY559) motifs and two PPPPP motifs in their binding partner (33). LATS1 contains two PPXY (373PPXY376 and 556PPXY559) motifs and two PPPPP motifs (235PPP240 and 247PPPPP251) within a P-stretch region (Fig. 2A). To examine which motif is responsible for its binding to YAP, we first performed a GST-pull down assay using cell lysate expressing YAP-HA and GST fusion proteins purified from bacteria expressing a series of LATS1 deletions. Only the region between aa. 526 and 655 in LATS1, which is immediate upstream of kinase domain and covers PPPY559 motif, interacted with YAP (Fig. 2B). Mutation of “Y” to “F” (phenylalanine) at aa.559 in LATS526-655 completely abolished its binding to YAP (Fig. 2C), indicating that PPPY559 motif in LATS1 is responsible for its interaction with YAP.

To map the regions in YAP responsible for its interacting with LATS1, we first performed a GST-pull down assay using cell lysate expressing LATS1-FLAG and GST fusion proteins purified from bacteria expressing a series of YAP deletions. As expected, only the region (aa.150-300) covering two WW domains in YAP bond to LATS1-FLAG (Fig. 2A, 2D). To determine which WW domain in YAP is responsible for its binding to LATS1, we created YAP150-300 mutants with mutations that disrupt single [ΔWW1 (W199A/P202A) or ΔWW2 (W258A/P261A)] or double [ΔWW1-2 (W199A/P202A/W258A/ P261A)] WW domains. Only disruption of both WW domains in YAP abrogated its binding to LATS1 (Fig. 2A, 2E). In conclusion, our results suggest that LATS1 binds to both WW domains of YAP through its PPPY559 motif.

Phosphorylation of YAP by LATS1—We used an IP kinase assay to examine whether YAP is a substrate of LATS1 kinase. LATS1-WT-FLAG or LATS1-KD-FLAG was transfected alone or together with Mst2-myc into COS-7 cells. LATS1-WT-FLAG or LATS1-KD-FLAG was precipitated from cell lysates expressing these constructs and subject to kinase assay using YAP-GST as a substrate. While precipitated LATS1-KD-FLAG has no activity toward YAP-GST in vitro, LATS1-WT-FLAG can phosphorylate YAP-GST with low activity (Fig. 3A). Since it has been shown recently that Mst2, the mammalian homolog of Drosophila Hippo, can phosphorylate and activate LATS1 kinase activity (34), we co-transfected LATS1 together with Mst2. As expected, co-transfection of Mst2-myc together with LATS1-KD-FLAG rather than LATS1-WT-FLAG significantly increases the phosphorylation of YAP-GST by precipitated LATS1-FLAG in vitro, whereas Mst2 alone was unable to phosphorylate YAP-GST (Fig. 3A). To confirm whether LATS1 can also phosphorylate
YAP in vivo, we transfected YAP-HA alone or together with LATS1-FLAG/Mst2-myc. LATS1 was able to phosphorylate YAP and caused a band shift of YAP-HA. This bandshift of YAP-HA is due to its phosphorylation by LATS1 because it was abolished after treatment of cell lysate with CIP (Fig. 3B). Therefore, YAP is a true substrate of LATS1 kinase.

Next, we would like to know whether other members of Ndr kinases can also phosphorylate YAP. First, we transfected WT or KD LATS1/2-FLAG or Ndr1/2-FLAG together with Mst2-myc into COS-7 cells. After precipitation by α-FLAG antibody, the in vitro kinase assay was performed using YAP-GST as substrates. Interestingly, all the precipitated WT LATS1/2 or Ndr1/2 rather than their KD counterparts were able to phosphorylate YAP in vitro (Fig. 3C). We also examined whether LATS/Ndr phosphorylate YAP in vivo by co-transfection of YAP-HA together with WT or KD LATS-FLAG/Mst2-Myc or Ndr-FLAG/Mst2-Myc. Significantly, co-transfection of WT other than KD of both LATS1 and LATS2 together with YAP-HA causes phosphorylation of YAP-HA. On the other hand, co-transfection of YAP-HA with WT or KD Ndr1 or Ndr2 had no effect on YAP-HA (Fig.3D), suggesting that YAP is the substrate of LATS rather than Ndr in vivo.

Identification of the LATS1 phosphorylation sites in YAP—To identify the sites in YAP phosphorylated by LATS1, we first performed in vitro kinase assays using precipitated WT or KD LATS1-FLAG as kinase and YAP GST fusion proteins purified from bacteria expressing different regions of YAP as substrates. All the YAP deletion fusion proteins including YAP1-150-GST, YAP150-300-GST, and YAP300-504-GST were significantly phosphorylated by wild-type LATS1 (Fig. 4A), suggesting that there are at least three phosphorylation sites in YAP. To further identify the phosphorylation sites, we created YAP deletions and used these deletion fusion proteins as substrates for in vitro kinase assay. YAP1-50-GST and YAP200-300-GST proteins were the only two fragments that were not phosphorylated by wild-type LATS1 (Fig. 4B). Since YAP1-100-GST and YAP150-300-GST were phosphorylated by LATS1, phosphorylation sites may be located in fragments of aa.50-100 and 150-200. By closer examination of all the potential Ser/Thr phosphorylation sites (3 sites in aa. 50-100 and 5 sites in aa. 150-200), we identify a phosphorylation motif, HXRXXS (H, histidine; X, any amino acid; R, arginine; S, serine) located at S61 (fragment 50-100) and S164 (fragment 150-200), that are present in both fragments (Fig. 4B). Further screening of this motif in YAP identified a total of 5 sites with this motif located at S61, S109, S127, S164, and S397 (Fig.4B and 4C). These 5 phosphorylation sites can perfectly explain the results of all the kinase assays (Fig. 4B, see asterisk as phosphorylation sites).

To confirm whether our hypothesis was true, we created YAP mutant fusion protein with single, double or triple phosphorylation site mutations and used these proteins as substrates for LATS1 kinase. Mutations of all the potential phosphorylation sites into alanines (As) (S61/109/127A) rather than a single (S61A, S109A or S127A) or double phosphorylation site (S109/127A) completely abolished the phosphorylation of YAP1-150-GST by LATS1. As expected, mutation of S164 (S164A) or S397 (S397A) in YAP150-300 and YAP300-504, respectively, also abolished their phosphorylation by LATS1, suggesting that all the 5 potential phosphorylation sites in YAP can be phosphorylated by LATS1. To further confirm whether YAP does contain 5 phosphorylation sites, we also created full-length YAP fusion proteins with all 5 phosphorylation site mutations (5×SA). As expected, mutations of all the phosphorylation sites (YAP-5×SA-GST) abolished the phosphorylation of YAP by LATS1 in vitro (Fig. 4E). This in vitro kinase assay result was further confirmed in vivo. Mutations of all phosphorylation sites (YAP-5×SA-HA) abrogated its bandshift/phosphorylation caused by over-expression of LATS1/Mst2 (Fig. 4F). In summary, these results clearly demonstrate that LATS1 phosphorylates YAP through 5 sites with a phosphorylation motif HXRXXS.

Identification of consensus phosphorylation sequence for LATS/Ndr kinase substrate—Sequence alignment of human (hYAP), mouse (mYAP) and Drosophila (dYAP=Yokie) YAP showed that, like hYAP, mYAP contains all the 5 phosphorylation sites identified in hYAP. However, dYAP/Yokie contains only two sites (S111 and S168) with HXRXXS motif corresponding to S109 and S127 in hYAP. A third
potential phosphorylation site (S250) with HXKXXS [K (lysine) replaces “R”] motif, which aligned with S164 phosphorylation site in hYAP, was also identified in dYAP (Fig. 5A) (see results below). To identify the consensus phosphorylation sequence for LATS1 substrates, we used the sequences surrounding the S127 phosphorylation site as a template and synthesized peptides with mutations at each conserved residue in the motif (Fig. 5B) to test their activity as the substrates of LATS1 kinase. Since “H”, “R”, and “K” all belong to the same group of positive charged amino acids and are often interchangeable in phosphorylation motifs, we first mutated “H” at -5 position into R (H122R) or K (H122K). We also mutated H122 into other amino acids such as N (asparagine, H122N) or A (H122A) to see whether they abolished their function as substrates (Fig. 5B). Significantly, like other mutations (H122N or H122A), mutation of “H” into “R” (H122R) or “K” (H122K) also completely eliminated its phosphorylation by LATS1 kinase (Fig. 5C), suggesting that “H” residue in the motif is essential for the phosphorylation of LATS1 substrate by LATS1. On the other hand, mutation of “R” at -3 position into “K” (R124K) or “H” (R124H) had only modest or no effect (Fig. 5C), whereas mutation of “R” into “A” (H125A) completely abolished its phosphorylation by LATS1. Since LATS1 is a Ser/Thr kinase, we also examined whether LATS1 can phosphorylate threonine residue using peptides with mutation from “S” to “T” (S127T). The phosphorylation of S127T by LATS1 dropped to 33% compared to its wild-type counterpart with serine as phosphorylation donor site. This suggests that LATS1 can phosphorylate both serine and threonine with preference for serine. This is consistent with the previous publications that most of Ser/Thr kinases have preference for their phosphorylation (35). Since other Ndr/LATS family members, such as Ndr1 and Ndr2, were also able to phosphorylate YAP in vitro (Fig. 3C), we tested whether they had the same consensus phosphorylation motif. Consistently, the same phosphorylation pattern was obtained for Ndr1 (Fig. 5D). According to the above results, we can conclude that the consensus phosphorylation sequence for LATS/Ndr family kinase substrates is: HXR/H/KXXS/T (Fig. 5E). We did an alignment of LATS/Ndr consensus phosphorylation sequences with those of other members of AGC kinase family. Surprisingly, all of the consensus sequences are very similar (Fig. 5E). Most of the AGC kinases except DMPK prefer S to T for phosphorylation. In addition, all of the consensus phosphorylation sequences for AGC kinase substrates contain an “R” at -3 position but are variable (R, K, H, L/I or X) at other positions. However, LATS/Ndr is the only kinase in AGC kinase family that has “H” in its consensus phosphorylation sequence at -5 and -3 positions (Fig. 5E).

Phosphorylation of YAP by LATS1 regulates its subcellular localization—Previous studies indicate that phosphorylation of YAP may affect its subcellular localization (36). To test whether phosphorylation of YAP by LATS also changes its subcellular localization, we transfected YAP-HA or YAP-5×SA-HA alone or together with LATS1-FLAG/Mst2-myc into COS-7 cells. Consistent with previous report (37), wild-type YAP was distributed in both the cytoplasm and nucleus. Although overexpression of LATS1 alone can sufficiently sequester YAP in the cytoplasm with low efficiency (data not shown), cotransfection of LATS1 together with Mst2 completely sequestered YAP in the cytoplasm in most of the cells examined (Fig. 6A). On the other hand, YAP-5×SA, a YAP mutant without any LATS1 phosphorylation sites, was mostly in the nucleus and remains unchanged after coexpression of LATS1/Mst2 in the cells. These results strongly suggest that the phosphorylation of YAP by LATS1 inhibits its translocation into the nucleus. We also examined whether overexpression of LATS1 affects subcellular localization of endogenous YAP by infection of HeLa cells with lentivirus expressing vector or LATS1 and Mst2 by protein fractionation. Consistent with results obtained from YAP-overexpression experiment stated above, endogenous YAP was mostly localized in both cytoplasm and nucleus. However, overexpression of LATS1/Mst2 caused exclusive localization of endogenous YAP in the cytoplasm. As cytoplasmic and nuclear protein fraction controls, α-tubulin and PCNA are localized in the cytoplasm and nucleus, respectively (Fig. 6B). We have also examined whether endogenous LATS affect subcellular localization of endogenous YAP.
As expected, after knockdown of LATS (LATS1 and LATS2) in HeLa cells, the endogenous YAP originally localized in both cytoplasm and nucleus was re-distributed exclusively into the nucleus (Fig. 6C). These results clearly demonstrate that phosphorylation of YAP by LATS1 inhibits its translocation to the nucleus, which leads to sequestration of YAP in the cytoplasm.

Identification of cellular genes regulated by YAP by microarrays—In Drosophila, Yokie/YAP was identified as an oncogene that promotes cell proliferation and inhibits apoptosis by up-regulation of cyclin E and dIAP transcription (15). Recently, mammalian YAP was also identified as an oncogene and over-expression of YAP caused transformation of MCF10A immortalized mammary cells. However, mammalian YAP has no effect on the expression of both cyclin E and cIAP (25), suggesting that the downstream genes mediating YAP-induced transformation in human cells may be different from those in Drosophila.

To identify the cellular genes up-regulated or down-regulated by YAP during transformation of mammary cells, we infected MCF10A cells with lentivirus expressing vector (MCF10A-control) or YAP-HA (MCF10A-YAP). GFP was also co-expressed by lentivirus vector and was used as a marker for infected cells. As shown by immunofluorescent images of infected cells, over 98% MCF10A were infected by lentivirus (Fig. 7A). Interestingly, infection of MCF10A by YAP rather than vector caused dramatic increase in YAP protein level and morphological changes (Fig. 7A and 7B). While MCF10A-control cells grow as epithelial-like cells, MCF10A-YAP cells lost cell-cell contact and displayed mesenchymal (fibroblast)-like morphology, a phenotype called EMT (epithelial-to-mesenchymal transition) (Fig. 7A).

To identify the genes regulated by YAP, we extracted RNAs from MCF10A-control and MCF10A-YAP cells and performed an expression profile analysis using Agilent 4×44K whole Human Genome Oligo (60mer) Array. In this microarray, many cellular genes with important biological functions have 3-10 probes (spots) on each array. After careful examination of signals from all the probes for each gene and statistic analysis (t-test) and GeneOntology analysis using GeneTool (see Experimental procedures for details), 17 up-regulated and 18 down-regulated genes associated with cell proliferation, cell death, cell migration, cell adhesion and EMT have been identified and are listed in Table 1. We used qRT-PCR to confirm some of the important genes significantly changed after over-expression of YAP. About 70% genes identified in microarray analysis as differentially expressed genes were also confirmed by qRT-PCR assays. As a positive control, YAP was 16-fold higher in MCF10A-YAP cells compared to MCF10A-control cells (Fig. 7C). For all the up-regulated genes examined, integrin β2 (ITGB2) is the gene that most significantly up-regulated (over 12-fold increase) by YAP. Many hormone or hormone-binding proteins, such as FGF1, IGFBP3, and PDGF β (PDGFB), were also enhanced after over-expression of YAP (Fig. 7C). Surprisingly, although YAP was originally identified as a transcription co-activator, the transcription of some of the tumor suppressors such as p57, RASSF4, prolactin (PRL), and BMP2, a member of TGFβ family, were significantly (3-10 fold) down-regulated by YAP (Fig. 7C).

Suppression of YAP-induced transcription regulation and cell transformation by LATS1—It has been shown that over-expression of Drosophila LATS inhibits cell proliferation and induces apoptosis by inhibiting Yokie trans-activation activity (15). Our experimental results demonstrate that LATS1 can phosphorylate YAP and inhibit its translocation to the nucleus, suggesting that phosphorylation of YAP by LATS1 may repress its transcription regulation of cellular genes in the nucleus. To test this possibility, we infected MCF10A-YAP cells with lentivirus expressing LATS1 and Mst2. Real-time qRT-PCR analysis of mRNA levels for genes regulated by YAP including FGF1, IGFBP3, integrin β2, PDGF β, BMP2, p57, PRL, and RASSF4 shows that over-expression of LATS1/Mst2 significantly inhibited YAP-induced transcription activation or repression of these cellular genes (Fig. 7C). We also tested whether LATS1 can inhibit YAP-induced MCF10A cell transformation using soft-agar assays. As shown in Figure 8A and 8B, no colony was formed on soft agar for MCF10A-control cells, further confirming that MCF10A cells are non-tumorigenic, immortalized mammary cells that do not display anchorage-independent growth. However, over-
expression of YAP caused MCF10A cell transformation as shown by the formation of many colonies on soft-agar (Fig. 8A and 8B). As expected, while infection of MCF10A-YAP cells with lentivirus expressing LATS1 alone significantly suppressed YAP-induced cell transformation, infection of MCF10A/YAP with lentiviruses expressing LATS1 together with Mst2 completely abolished cell transformation (Fig. 8A and 8B). On the other hand, YAP-5×SA lacking LATS phosphorylation sites had higher transformation activity (more colonies formed) and became resistance to LATS1 or LATS1/Mst2-induced transformation inhibition (Fig. 8A, 8B).

DISCUSSION

Although tremendous progress has been made toward our understanding the emerging Hippo-LATS/Warts tumor suppressor signaling pathway in the regulation of cell growth, cell proliferation, and cell death in Drosophila, little is known about this pathway in mammals. In this study, we have provided convincing evidence that human LATS1 is a negative regulator of YAP/Yokie. LATS1 together with Mst2 can significantly inhibit both YAP-induced transcription regulation and YAP-induced cell transformation of mammary immortalized cells. Consistent to our findings, YAP was also recently found to be inhibited by Drosophila LATS/Warts and human LATS2 through phosphorylation and play important roles in the regulation of animal organ size and cell contact inhibition (38-39). Together with the finding that Mst1/2 can phosphorylate and activate LATS1/2 (34), we can conclude that part of the Hippo-LATS/Warts signaling pathway, Mst1/2→LATS1/2→YAP, is conserved in both Drosophila and mammals.

LATS is the central player in the Hippo-LATS/Warts signaling pathway. Previous studies have shown that both fly and mammalian LATSs are involved in tumorigenesis by regulating cell proliferation, cell growth, and apoptosis (6-7, 14, 22). However, the signaling pathways by which LATS suppresses tumor growth are not fully understood. Since LATS is a kinase and the kinase activity is essential for its tumor suppressing and other functions (6-7, 28-31), identification of the kinase substrates of LATS is crucial to elucidate the biological and biochemical functions of LATS. So far, Yokie is the only protein identified as the substrate of LATS kinase in Drosophila (15). However, how LATS phosphorylation regulates Yokie activity and whether mammalian LATS1/2 and their homologs Ndr1/2 also phosphorylate mammalian Yokie homolog, YAP, remains to be explored. In this study, we have shown for the first time that human LATS1 and LATS2 can physically interact with and phosphorylate YAP in vitro and in vivo, whereas LATS homologs Ndr1 and Ndr2 were unable to bind to YAP and could only phosphorylate YAP in vitro. We have also mapped the domains in LATS1 and YAP responsible for their interactions. We found that the PPXY in LATS1 and both WW domains in YAP are essential for their interactions (Fig. 2A-E).

Sequence analysis shows that there is another PPXY motif located at aa.373-376 (373PPPY376) in LATS1 (Fig. 2A). However, this motif is not conserved and cannot be found in other LATS such as LATS2 or Drosophila LATS. Therefore, it is possible that the structure surrounding this motif prohibits it from binding to YAP. By examining the sequence of Ndr1/2, we could not find any PPXY motif. This explains why LATS rather than Ndr could bind to YAP. However, the kinase domain of LATS has high homolog to that of Ndr, suggesting that they may phosphorylate the same substrate. As expected, both Ndr1 and Ndr2 were able to phosphorylate YAP in vitro. Since Ndr was unable to interact with YAP, they were unable to phosphorylate YAP in vivo. Therefore, LATS1 and LATS2, but not Ndr1 and Ndr2, are the true kinases phosphorylating YAP.

Although Yokie was found to be phosphorylated by Drosophila LATS, the phosphorylation sites in Yokie have not been identified (15). Identification of phosphorylation sites in Yokie/YAP is very important to elucidate their roles in Hippo-LATS/Warts pathway. By making a series of deletions and mutations, we have identified 5 phosphorylation sites with the same HXRXXS motif in YAP. Importantly, three out of these 5 phosphorylation sites are also conserved in Drosophila Yokie. By using a peptide kinase assay, we have also identified the consensus phosphorylation sequence, HXR/H/KXXX/T, for both LATS1/2 and Ndr1/2 kinase substrates. Interestingly, Dbf2, a yeast homolog of LATS, was found to preferentially...
phosphorylate peptides containing RXXS motif (40), which covers part of our consensus phosphorylation sequence. These findings strongly suggest that all the members of LATS/Ndr kinase family in all organisms may have the same phosphorylation motifs. By searching for proteins having this motif using ScanSite (http://scansite.mit.edu/), we were able to identify many potential substrates of LATS/Ndr with this motif. YAP/Yokie is the only kinase substrate identified so far for LATS. Identification of the consensus phosphorylation sequence will greatly facilitate our efforts to find more kinase substrates of Ndr/LATS kinases that may be important in mediating the emerging Hippo-LATS/Warts pathway and carrying out various biological functions such as tumor suppression, organ size control, and neuron tilling, etc. Moreover, this consensus phosphorylation sequence is very similar to those of other AGC kinase family members (Fig. 5E). Due to the similarity of their consensus phosphorylation sequences, many members of AGC kinase family can often phosphorylate the same substrate. For example, Akt, p70S6K and RSK1 AGC kinases were able to phosphorylate Tau on the same phosphorylation site (41). However, LATS and Ndr kinases are very unique and are the only two kinase kinases that have “H” at -5 and -3 positions of its consensus phosphorylation sequence. Specifically, “H” at -5 position of the consensus phosphorylation motif is crucial for their substrates to be phosphorylated (Fig. 5C and 5D). Ndr family kinases have a unique feature that is not found in any other AGC kinases. They have an insert of about 30-60 amino acids located between subdomains VII and VIII of the kinase domain. This unique feature possibly distinct them from other AGC kinases and restricts their substrate specificity. This may be also why only one kinase substrate of LATS has been identified so far.

Interestingly, one of the 5 phosphorylation sites in YAP, S127, was also found to be phosphorylated by another AGC kinase Akt1/PKB (35). Akt1/PKB has a consensus phosphorylation sequence RXRXXXS. Although more than over 100 substrates have been identified for Akt/PKB (42), YAP is the only substrate that has HXRXXXS phosphorylation motif (35). We also tested the possibility of whether LATS/Ndr and Akt/PKB share their substrates as was found in other AGC family kinases. Our peptide kinase assays clearly demonstrate that “H” residue in the HXRXXXS motif is crucial and replacement of “H” with “R”, which mimic Akt/PKB consensus phosphorylation motif, completely eliminated its phosphorylation by LATS1/Ndr1 (Fig. 5C and 5D). Therefore, LATS/Ndr and Akt/PKB do not share their substrates. Although our findings and the results shown in Drosophila genetic studies clearly demonstrated that YAP is the true substrate of LATS, it remains to be tested whether Akt is also the kinase phosphorylating YAP in our experimental system.

Although Yokie/YAP was found to be phosphorylated by Drosophila LATS, the significance of this phosphorylation on Yokie/YAP function is still unknown. In this study, we provided convincing evidence that phosphorylation of YAP by LATS1 sequestered YAP in the cytoplasm, which would prohibit it from functioning as a transcription regulator in the nucleus. Over-expression of LATS1/Mst2, which phosphorylates YAP, caused retention of YAP in the cytoplasm, whereas mutations of phosphorylation sites in YAP (YAP-5×SA) allowed YAP to be localized mostly in the nucleus and resistant to LATS1-induced effect on its subcellular localization (Fig. 6A). Contrary to the effect of LATS1 on YAP, phosphorylation of tyrosine 433 (Y433) in YAP by Yes tyrosine kinase promotes its translocation to nucleus to repress Runx2-induced transcription activation (43). Therefore, phosphorylation of YAP plays a very important role in regulating its function. The phosphorylated S127 in YAP has been shown to be the binding site for 14-3-3 (35). Therefore, it is likely that phosphorylation of YAP by LATS1 increases its interaction with cytoplasmic protein 14-3-3. However, it remains to be explored whether phosphorylation of all the phosphorylation sites of YAP by LATS1 is essential for the full inhibition of YAP function.

Both Drosophila and mammalian YAP/Yokie has been identified as an oncogene important for tumorigenesis (15, 25-26). Over-expression of Yokie/YAP caused enhanced cell proliferation, reduced apoptosis, and tumor formation in Drosophila and mice and transformation of immortalized mammary cells (15, 25). In addition, our studies and previous publications also showed that over-expression of YAP induced many
cellular changes such as disrupted actin cytoskeletal organization and cell-cell adhesion, increased cell migration, and EMT (Hao et al., unpublished results, Fig.7A, 25), all of which are the phenotypes for invasive tumor cells. Although up-regulation of cycle E and DIAP transcription has been proposed to be partially responsible for Drosophila Yokie/YAP-induced tumor formation, induction of cyclin E and diAP1 cannot fully account for all the phenotypes caused by over-expression of Yokie/YAP in Drosophila (11). In mammalian cells, it has been shown that cycle E and cIAP1 are not induced after over-expression of YAP (25). Although YAP was originally identified as transcription co-activator and regulates many transcription factors such as PEBP2, p73, P53-BP-2, and TEAD/TEF (36, 44-46), the downstream genes mediating YAP-induced cell transformation and many cellular changes such as EMT remain to be identified. By using Whole Human Genome Oligo Microarray, we have successfully identified many genes regulated by YAP. These genes are involved in the regulation of cell proliferation, cell death, cytoskeletal organization, cell migration and cell adhesion. FGF1 and PDGFβ activated by YAP are growth hormones that induce increased cell proliferation, cell migration and EMT (47-48), suggesting that YAP may up-regulate growth hormone transcription to induce cell transformation. Interestingly, we have also found that over-expression of YAP dramatically induce β2 Integrin, a member of integrin family. Integrins are proteins essential for cell adhesion, cell migration, and anchorage-dependent cell proliferation and cell survival (49-50). It has been shown that over-expression of β4 integrin caused anchorage-independent growth and transformation of rodent fibroblast, whereas loss of β4 integrin in breast carcinoma cells abrogated anchorage-independent growth and tumor formation in nude mice (51). Integrin β2 is cell surface protein predominantly expressed in leukocyte and present in low level in other tissues (49). Like other integrins, β2 integrin has also been shown to be involved in cell-cell and cell-matrix adhesions of leukocytes (49). However, whether β2 integrin is involved in tumorigenesis has not been reported so far. This is the first report that integrin β2 is significantly induced in mammary cells after cell transformation. Therefore, β2 integrin may be a very important downstream protein mediating YAP-induced anchorage-independent growth and transformation of mammary cells. It will be very interesting to examine whether over-expression of β2 integrin can also transform MCF10A mammary cells or down-regulation of integrin in MCF10A-YAP cells can block their anchorage-independent growth and transformation or other cellular changes.

Surprisingly, we have also identified many genes significantly suppressed by YAP. These genes are usually negative regulators of cell proliferation or tumor invasion. For example, p57 and RASSF4 have been shown to be tumor suppressors that inhibit tumor cell proliferation and tumor formation and induce apoptosis (52-53), whereas PRL has been shown to suppress cell proliferation and tumor invasion and EMT (54). Therefore, down-regulation of these genes by YAP will induce increased cell proliferation and cell migration, reduced apoptosis, and formation of EMT. In fact, it has also been previously shown that over-expression of YAP inhibited Runx2-mediated activation of osteocalcin transcription (43). Therefore, YAP may also cause cell transformation and enhanced cell proliferation and tumor invasion by inhibiting transcription of several negative regulators of cell proliferation and cell migration and inducers of apoptosis. Most importantly, our studies strongly suggest that although both mammalian and Drosophila YAP/Yokie can induce tumor formation by regulating transcription of downstream genes they may use different pathways to accomplish their function as oncogene. Further characterization of these downstream genes of YAP will have great impact on our understanding the signal transduction pathway of this emerging Hippo-LATS/Warts pathway during tumorigenesis.

In conclusion, we have provided convincing evidence that, human tumor suppressor LATS is a negative regulator of oncogene YAP. LATS1 inactivates YAP oncogenic function by phosphorylating YAP, which inhibits its translocation into nucleus to regulate cellular genes important for cell proliferation, cell death, and cell migration. Most significantly, by using YAP phosphorylation sites as a template, we were able to identify HXR/H/KXXS/T as the consensus phosphorylation sequence of LATS/Ndr kinase.
substrate. Given the fact that Hippo-LATS/Warts tumor suppressor signaling pathway and Ndr/LATS kinases are very important not only in tumorigenesis but also in various biological processes, our research findings will have profound impact on our understanding the molecular mechanism and the signal transduction pathway underlining their biological functions.

FOOTNOTES
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**FIGURE LEGENDS**
Figure 1. Interaction of LATS/Ndr with YAP. A. Co-IP of LATS1 and YAP. LATS1-FLAG and YAP-HA were transfected alone or together into COS-7 cells. About 500 μg of protein lysate were precipitated with 2 μg of α-HA (Y11) polyclonal antibody. Precipitated proteins were subject to western blot (WB) with α-FLAG (M2) monoclonal antibody. About 10 μg of protein lysate were also subject to WB with indicated antibodies to examine whether LATS1-FLAG and YAP-HA are expressed in COS-7 cells. B. Interaction of endogenous LATS1 and YAP in HeLa cells. About 500 μg of HeLa cell lysate were incubated with rabbit IgG (control) or α-YAP polyclonal antibody. The precipitated YAP immunocomplexes were subject to WB with α-LATS1 (Y03) polyclonal antibody along with 50 μg of HeLa cell lysate (1/10 input) precipitated with α-LATS1 antibody. C. Interaction of YAP with LATS/Ndr in vitro. About 10 μg of GST (control) or YAP-GST were incubated with 100 μg of cell lysate from LATS1/2 over-expressing cells or 20 μg of cell lysate from Ndr1/2 over-expressing cells. GST fusion proteins were precipitated with glutathione-sepharose beads and subject to WB with α-FLAG antibody along with 1/10 amounts of cell lysate used for GST pull-down assays (1/10 input). The membrane was stained with 1% Ponseau S to visualize the GST fusion proteins used in the GST-pull-down assays.

Figure 2. Functional domains of LATS1 and YAP responsible for their interactions. A. Schematic diagram of LATS1 and YAP structures. B. Interaction of YAP with LATS1 deletions in vitro. GST or LATS1 GST fusion proteins with a series of deletions were incubated with 20 μg of cell lysate from cells expressing YAP-HA. The procedures for GST-pull-down assays were as described in Figure 1C. C. Identification of motif in LATS1 responsible for its interaction with YAP. For GST pull-down assays, GST, wild-type LATS526-655-GST or LATS526-655-GST with mutation from “Y” in the PPPY559 motif to “F” (Y559F) were incubated with cell lysate from YAP-HA over-expressing cells. D. Interaction of LATS1 with YAP deletions in vitro. GST or YAP GST fusion proteins with a series of deletions were incubated with 100 μg of cell lysate from cells expressing LATS1-FLAG. The procedures for GST-pull-down assays were as described in Figure 1C. E. Interaction of LATS1 with YAP with WW domain mutations in vitro. For GST-pull-down assays, GST, wild-type YAP150-300-GST or YAP150-300-GST with single (ΔWW1 or ΔWW2) or double (ΔWW1-2) WW domain mutations were incubated with LATS1-FLAG lysate and processed as described in Figure 1C.

Figure 3. Phosphorylation of YAP by LATS/Ndr. A. Phosphorylation of YAP by LATS1 in vitro. COS-7 cells were transfected with plasmids expressing WT or KD LATS1-FLAG alone or together with Mst2-myc. LATS1-FLAG was then immunoprecipitated with α-FLAG antibody along with Protein G beads. The precipitated LATS1 was then used for in vitro kinase assay in the presence of γ-ATP using 2 μg of YAP-GST as substrate. The reaction mix was subsequently subject to 10% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and stained with 1% Ponseau S to visualize YAP-GST substrates (bottom band), and exposed to film (bands with 32P). B. Phosphorylation of YAP by LATS1 in vivo. YAP-HA was transfected alone or together with LATS1-FLAG and Mst2-myc into COS-7 cells. Cell lysate from cells transfected with YAP-HA/LATS1-FLAG/Mst2-Myc was treated with CIP to dephosphorylate phosphorylated YAP-HA. The protein lysates were subject to WB with indicated antibodies. C. Phosphorylation of YAP by LATS/Ndr kinases in vitro. WT or KD LATS-FLAG or Ndr-FLAG were co-transfected with Mst2-myc into COS-7 cells. IP-kinase assays were performed as described in Figure 3A. D. Phosphorylation of YAP by LATS/Ndr kinases in vivo. WT or KD LATS-FLAG or Ndr-FLAG was co-transfected with YAP-HA and Mst2-myc into COS-7 cells. The cell lysates were subject to WB with indicated antibodies.

Figure 4. Mapping of the phosphorylation sites in YAP. A. Phosphorylation of YAP deletion proteins by LATS1. A series of YAP fusion proteins with deletions were used as substrates for in vitro kinase assay using LATS1-WT-FLAG activated by Mst2 or LATS1-KD-FLAG. Potential phosphorylation sites were shown in the schematic graph. Black bars in B indicate that these two fusion
proteins were not phosphorylated by LATS1. C. Alignment of the sequences of phosphorylation sites in YAP. Conserved residues are highlighted. D. Phosphorylation of YAP mutants by LATS1. YAP fusion proteins with single (S61A, S109A, S127A, S164A), double (S109/127A) or triple (S61/109/127A) mutations were used as substrates for in vitro kinase assay using activated LATS1 kinase. E. Phosphorylation of YAP with phosphorylation site mutations by LATS1 in vitro. In vitro kinase assays were carried out using activated LATS1 kinase, and WT and 5×SA (“S” in all the 5 phosphorylation sites were mutatated into “A”) YAP as substrates. F. Phosphorylation of YAP with phosphorylation site mutations by LATS1 in vivo. YAP-HA or YAP-5×SA-HA was transfected alone or together with LATS1/Mst2 into COS-7 cells. About 10 μg of protein lysates were used for WB with indicated antibodies.

Figure 5. Identification of consensus phosphorylation sequence for LATS substrates. A. Alignment of YAP phosphorylation site sequences from different organism. hYAP, human YAP; mYAP, mouse YAP; dYAP, Drosophila YAP (Yokie). B. Peptides sequences surrounding YAP-S127 and its mutants. The conserved residues in WT YAP-S127 and each mutated residue were in boldface. Amino acid position for each residue in YAP was shown on the top. Phosphorylation site at S127 is regarded as position 0 in the sequence. C. In vitro phosphorylation of peptide substrates by LATS1. In vitro kinase assays were carried out using immunoprecipitated, activated LATS1 and various peptide substrates in the presence of γ-ATP32. Circle P81 filter papers were used for phosphorylated peptide capture and radioactivity quantification. Phosphorylation activity by LATS1 is calculated as percentage of CPM for each mutant peptide compare to CPM of WT peptide. Error bars equal ±SD of two independent kinase assays for each peptide substrates. D. In vitro phosphorylation of peptide substrates by Ndr1. The in vitro kinase assays were carried out as described above in C except that Ndr1 was used as the kinase for the assay. E. Sequence alignment of AGC family kinases. Conserved residues were highlighted. Phosphorylation site (S/T) was regarded as position 0. Note that only DMPK prefer T to S for phosphorylation.

Figure 6. Phosphorylation of YAP by LATS1 enhances its localization in the cytoplasm. A. Immunofluorescent staining of YAP and LATS1. YAP-HA or YAP-5×SA-HA was transfected alone or together with LATS1-FLAG/Mst2-Myc into COS-7 cells. Transfected cells were fixed with 3.7% formaldehyde and permeabilized in 0.2% Triton X-100 in PBS. For cells transfected with YAP-HA or YAP-5×SA-HA alone, the fixed cells were stained with Y11 α-HA polyclonal antibody followed by AF555 α-rabbit IgG (red). For cells transfected with YAP-HA or YAP-5×SA-HA together with LATS1-FLAG/Mst2-myc, the fixed cells were co-stained with α-HA polyclonal antibody (YAP-HA or YAP-5×SA-HA) together with α-FLAG (LATS1) monoclonal antibody followed by AF555 α-rabbit IgG (red) and AF488 α-mouse IgG (green). B. Over-expression of LATS1/Mst2 induces re-localization of endogenous YAP in cytoplasm. HeLa cells were infected with LATS1 and Mst2 lentiviruses. Cytoplasmic and nuclear protein fractions were subject to WB to examine the subcellular localization of endogenous YAP using α-YAP polyclonal antibody. α-tubulin and PCNA were used as cytoplasm and nuclear markers, respectively, for the purity of the protein fractionation. The same protein lysates were also used to examine the expression of LATS1-myc and Mst2-FLAG by WB using α-Myc (9E10) and α-FLAG (M2) antibodies, respectively. C. Subcellular localization of endogenous YAP after knockdown of LATS by siRNA. HeLa cells were transfected with control-siRNA or LATS1-siRNA together with LATS2-siRNA (10 nM). To confirm LATS knockdown, four days after transfection, the proteins were extracted and subject to WB using rabbit α-LATS1 (Y03) or α-LATS2 (BL2213) polyclonal antibody. Cells from the same transfection were re-plated onto a coated coverslip and subject to immunofluorescent staining as described in A using rabbit α-YAP polyclonal antibody (H-125) followed AF488 α-rabbit IgG.

Figure 7. Inhibition of YAP-induced transcription regulation by LATS1. A. Morphology of MCF10A cells after over-expression of YAP. MCF10A cells were infected with lentivirus expressing WPI vector
(control) or YAP. Cell morphology was examined under Nikon TE-2000 inverted fluorescent microscope using white light (left) or fluorescent light (right) at 10× magnification. GFP expressed from lentivirus vector was used as marker for infection efficiency. B. Expression of YAP after infection of MCF10A with YAP lentivirus. MCF10A-control cells or duplicate MCF10A-YAP (YAP-1 and YAP-2) cells were lysed and subject to WB for YAP-HA expression using α-HA antibody. β-actin was used as an internal loading control. C. Inhibition of YAP-induced changes in cellular gene transcription by LATS1. Real-time qRT-PCR was performed to examine selected cellular gene expression in MCF10A-control, YAP-overexpressing, and YAP+LATS1+Mst2 expressing MCF10A cells. The relative mRNA levels in MCF10A-YAP and MCF10A-YAP+LATS1+Mst2 cells to those in MCF10A-control cells were presented here. The mean and standard deviation (SD) were calculated from Ct values of triplicate real-time RT-PCR reactions for each RNA sample.

Figure 8. Suppression of YAP-induced transformation by LATS1. A. Anchorage-independent growth in soft-agar. About 5×10^4 MCF10A cells infected with lentivirus expressing vector (control), YAP, YAP+LATS1, YAP+LATS1+Mst2, YAP-5×SA, YAP-5×SA+LATS1, and YAP-5×SA+LATS1+Mst2 were grown on soft agar in a well of 6-well plate for 21 days. Representative wells stained with 0.005% crystal violet are shown. B. Quantification of soft agar assays. Error bars equal mean±SD of three independent experiments.
Table 1. List of cellular genes up-regulated and down-regulated by YAP

| Gene Accession Number | Name                  | Description                                         | Function                                      |
|-----------------------|-----------------------|-----------------------------------------------------|-----------------------------------------------|
| **Up-regulated genes**|                       |                                                     |                                               |
| ATF3                  | NM_001040619          | Activating transcription factor 3                   | Cell death, cell migration                     |
| BIRC5                 | NM_0010227            | Baculoviral IAP repeat-containing 5                 | Cell death                                    |
| CORO2B                | NM_006091             | Coronin, actin-binding protein, 2B                  | Cell adhesion                                 |
| DSC3                  | NM_024423             | Desmocollin 3                                      | Cell adhesion                                 |
| EXT1                  | NM_000127             | Exostoses (multiple) 1                             | Cell cycle                                    |
| FGF1                  | NM_000800             | Fibroblast growth factor 1 (acidic)                | Cell proliferation, cell migration, EMT       |
| IGFBP3                | NM_001013398          | Insulin-like growth factor binding protein 3       | Cell proliferation, cell death                |
| IL4                   | NM_000589             | Interleukin 4                                       | Cell proliferation, cell migration            |
| IL10                  | NM_000572             | Interleukin 10                                      | Cell proliferation, cell death, cell migration|
| ITGB2                 | NM_000211             | Integrin β2                                        | Cell cycle, cell death                        |
| LCK                   | NM_005356             | Lymphocyte-specific protein tyrosine kinase        | Cell cycle, cell death                        |
| PDGFB                 | NM_002608             | Platelet-derived growth factor beta                | Cell proliferation, cell migration, EMT       |
| PLK2                  | NM_006622             | Polo-like kinase 2                                  | Cell proliferation                            |
| PTSCR                 | NM_008923             | Protein tyrosine phosphatase, receptor type C      | Cell proliferation, cell death                |
| PTPRF                 | NM_002840             | Protein tyrosine phosphatase, receptor type F      | Cell adhesion                                 |
| PPP1R9B               | NM_017650             | Protein phosphatase 1, regulatory subunit A         | Cell adhesion                                 |
| **Down-regulated genes**|                      |                                                     |                                               |
| BMP2                  | NM_001200             | Bone morphogenetic protein 2                       | Cell proliferation                            |
| CXCL1                 | NM_001511             | Chemokine (X-X-C motif) ligand 1                   | Cell proliferation                            |
| CDKN1C                | NM_000076             | Cyclin-dependent kinase inhibitor 1C               | (p57,Kip2)                                   |
| EMCN                  | NM_016242             | Endomucin                                          | Cell adhesion                                 |
| IL1A                  | NM_000575             | Interleukin 1, alpha                                | Cell proliferation, cell death                |
| IL1B                  | NM_000576             | Interleukin 1, beta                                 | Cell proliferation, cell death                |
| IL6                   | NM_000600             | Interleukin 6                                      | Cell proliferation, cell death                |
| IL11                  | NM_000641             | Interleukin 11                                     | Cell proliferation                            |
| ITGA5                 | NM_002205             | Integrin, alpha 5                                  | Cell adhesion                                 |
| JAM2                  | NM_021219             | Junctional adhesion molecule 2                     | Cell adhesion                                 |
| LSP1                  | NM_001013254          | Lymphocyte-specific protein 1                      | Cell migration                                |
| PRL                   | NM_000948             | Prolactin                                          | Cell proliferation, cell migration, EMT       |
| RASSF4                | NM_032023             | Ras association domain family 4                    | Cell proliferation                            |
| SYK                   | NM_003177             | Spleen tyrosine kinase                             | Cell proliferation, cell migration, EMT       |
| TIMP1                 | NM_003254             | TIMP metalloproteinase inhibitor 1                 | Cell proliferation, cell migration, EMT       |
| TLR2                  | NM_003264             | Toll-like receptor 2                                | Cell death                                    |
| TNFAIP6               | NM_007115             | Tumor necrosis factor, alpha-induced protein 6     | Cell adhesion                                 |
| WASPPIP               | NM_003387             | Wiskott-Aldrich syndrome protein interacting protein| Cell migration                                |
Hao et al. Figure 1

A

|        | LATS1-FLAG | YAP-HA | IP-HA-FLAG | WB-(x)-FLAG | WB-(x)-HA | WB-(x)-FLAG |
|--------|------------|--------|------------|-------------|-----------|-------------|
| Control| +          | +      | +          | +           | +         | +           |

B

- LATS1
- YAP
- Heavy chain

C

- LATS1-FLAG
- LATS2-FLAG
- Ndr1-FLAG
- Ndr2-FLAG

WB: anti-LATS1

Ponceau S staining
Hao et al. Figure 3

A

| LATS1-WT-FLAG | - | + | - | - | + | - |
| LATS1-KD-FLAG  | - | - | + | + | + | + |
| Mst2-Myc       | - | - | - | - | - | + |
| YAP-GST        | + | + | + | + | + | + |

B

| LATS1-FLAG  | - | + | + |
| Mst2-Myc    | - | + | + |
| YAP-HA      | + | + | + |
| CIP         | - | - | + |

C

| LATS1-FLAG | WT | KD |
| LATS2-FLAG | WT | KD |
| Ndr1-FLAG  | WT | KD |
| Ndr2-FLAG  | WT | KD |

D

| Mst2-Myc   | + | + | + | + |
| YAP-HA     | + | + | + | + |

WB \(\alpha\)-HA

WB \(\alpha\)-FLAG

WB \(\alpha\)-Myc
Hao et al. Figure 4

A

LATS1-FLAG

|       | YAP1-150-GST | YAP150-300-GST | YAP300-504-GST |
|-------|--------------|----------------|----------------|
| WT    |              |                |                |
| KD    |              |                |                |

32p

Ponceau S staining

IgG heavy chain

B

|   | Kinase Assay |
|---|--------------|
| 1 | +            |
| 2 | +            |
| 3 | +            |
| 4 | -            |
| 5 | +            |
| 6 | +            |
| 7 | +            |
| 8 | +            |
| 9 | +            |

C

YAP-S61 QIVHEVTD
YAP-S109 PKSKEQATSTD
YAP-S127 TPQHVPAHSSP
YAP-S164 TAQHLQQSFE
YAP-S397 GTYHREDSTD

D

E

YAP-GST

F

Msi2-Myc

LATS1-FLAG

YAP-5xSA-HA

YAP-HA

α-HA

α-FLAG

α-Myc
Hao et al. Figure 7

A

B

C

Control  YAP-1  YAP-2

YAP  Actin

C

Relative to control (fold)

CON  YAP  YAP+ LATS1+ Mnt2

YAP  FGF1  IGFBP3

Relative to control (fold)

CON  YAP  YAP+ LATS1+ Mnt2

ITGB2  PDGF2  BMP2

Relative to control (fold)

CON  YAP  YAP+ LATS1+ Mnt2

p57  PRL  RASSF4

Relative to control (fold)

CON  YAP  YAP+ LATS1+ Mnt2
Hao et al., Figure 8

A

Control
YAP
YAP+LATS1
YAP+LATS1+Mst2
YAP-5xSA
YAP-5xSA+LATS1
YAP-5xSA+LATS1+Mst2

B

Calories per plate

Control YAP YAP+LATS1 YAP+LATS1+Mst2 YAP-5xSA YAP-5xSA+LATS1 YAP-5xSA+LATS1+Mst2

25
Tumor suppressor LATS1 is a negative regulator of oncogene YAP
Yawei Hao, Alex Chun, Kevin Cheung, Babak Rashidi and Xiaolong Yang

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