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Cancer Research

Src Inhibits the Hippo Tumor Suppressor Pathway through Tyrosine Phosphorylation of Lats1

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Abstract

The Hippo pathway regulates cell proliferation, apoptosis, and stem cell self-renewal, and its inactivation in animal models causes organ enlargement followed by tumorigenesis. Hippo pathway deregulation occurs in many human cancers, but the underlying mechanisms are not fully understood. Here, we report tyrosine phosphorylation of the Hippo pathway tumor suppressor LATS1 as a mechanism underlying its regulation by cell adhesion. A tyrosine kinase library screen identified Src as the kinase to directly phosphorylate LATS1 on multiple residues, causing attenuated Mob kinase activator binding and structural alteration of the substrate-binding pocket in the kinase domain. Cell matrix adhesion activated the Hippo pathway effector transcription coactivator YAP partially through Src-mediated phosphorylation and inhibition of LATS1. Aberrant Src activation abolished the tumor suppressor activity of LATS1 and induced tumorigenesis in a YAP-dependent manner. Protein levels of Src in human breast cancer tissues correlated with accumulation of active YAP dephosphorylated on the LATS1 target site. These findings reveal tyrosine phosphorylation of LATS1 by Src as a novel mechanism of Hippo pathway regulation by cell adhesion and suggest Src activation as an underlying reason for YAP deregulation in tumorigenesis. *Cancer Res; 77(18); 4868–80. ©2017 AACR.*

Introduction

In multicellular organisms, the relative size of each organ is precisely controlled. However, the underlying mechanism is largely obscure. In recent years, the Hippo signaling pathway was found to play an evolutionarily conserved role in organ size control (1, 2). Mutation of this pathway leads to dramatic organ enlargement in *Drosophila* and mice. Biochemical and genetic analysis determined a linear pathway in which the Mst1 and Mst2 kinases (referred to as Mst, the *Drosophila* Hippo homologs) in complex with a scaffold protein Sav1, phosphorylate, and activate the Lats1 and Lats2 kinases (referred to as Lats, the *Drosophila* Wts homologs), which then in turn phosphorylate and inactivate a transcription coactivator Yes-associated protein (YAP) and its paralog transcriptional coactivator with PDZ-binding motif (TAZ; both are *Drosophila* Yki homologs; refs. 1, 2). Lats is also activated by Mob1a and Mob1b proteins (collectively referred to as Mob) through physical interaction (3). Phosphorylation by Lats inactivates YAP and TAZ through cytoplasmic retention and protein degradation (4–9). Therefore, when the Hippo pathway is inactive, dephosphorylated YAP translocates to the cell nucleus and binds to transcription factors such as TEADs to promote gene expression (2, 10). As a result, cell proliferation and stem cell self-renewal are enhanced and apoptosis is inhibited, resulting in tissue overgrowth and organ size enlargement.

The growth-promoting activity of YAP is precisely controlled under physiological conditions. However, the regulating mechanisms are apparently taken down by various means in human cancers, resulting in YAP activation and tumorigenesis (2). For instance, mutations of the Hippo pathway upstream components *NF2* and *GNAQ*/*GNA11* are major causes of neurofibromatosis 2 and uveal melanoma, respectively (11–13). In addition, amplification of the YAP gene locus has been observed in cancers such as hepatocellular carcinoma (14–17). However, these genetic variations are responsible for only a small fraction of YAP activation in cancer while the other cases remain unexplained on the molecular level. Nevertheless, the potent tumorigenic activity of YAP has been demonstrated in various animal models (18–21). For example, liver-specific knockout of *Mst1/2* or transgenic expression of *YAP* potently induces liver tumorigenesis following liver enlargement. In addition, YAP activation also plays an important role in cancer stem cells, tumor microenvironment, and tumor relapse (22–24). These findings highlight the important roles of aberrant YAP activation in cancer initiation and progression. Thus, a better understanding of Hippo pathway regulation and abnormality would facilitate cancer prevention and treatment.

The Hippo pathway transduces some unique extracellular signals to the cell nucleus, for example, mechanical stresses such as cell adhesion, matrix stiffness, and shear stress; hormonal

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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signaling mediated by a myriad of G-protein–coupled receptors (GPCRs); and cellular energy stress sensed by the AMP-activated protein kinase (2, 25). However, mechanisms transducing mechanical signals to the Lats kinase are not well understood, which leaves a major gap in the Hippo pathway. Here, we found that Src kinase, which is activated by cell adhesion, directly phosphorylates Lats1 on tyrosine residues, resulting in Lats1 inhibition, followed by YAP activation. Furthermore, we demonstrate that aberrant Src activation inactivates Lats1 tumor suppressor and induces tumorigenesis in a YAP-dependent manner. Importantly, human breast cancer samples with elevated Src protein level clearly exhibit accumulation of dephosphorylated active YAP. Our findings reveal tyrosine phosphorylation of Lats1 by Src as a novel mechanism mediating Hippo pathway regulation by cell adhesion and Hippo pathway deregulation in tumorigenesis.

Materials and Methods

Cell culture

HEK293T, NIH-3T3, MDA-MB-231, and HeLa cells were gifts from Dr. Kun-Liang Guan's laboratory at the year 2012. BT-474, MCF10A, and DLD-1 cells were purchased (year 2009) from ATCC, where they were characterized by DNA finger printing. Cell line authentication was not done in the lab. HEK293T, NIH-3T3, HeLa, BT-474, and MDA-MB-231 cells were cultured in DMEM (Life Technologies) containing 10% FBS (Life Technologies) and 50 μg/mL penicillin/streptomycin (P/S). MCF10A cells were cultured in DMEM/F12 (Life Technologies) supplemented with 5% horse serum (Life Technologies), 20 ng/mL EGF, 0.5 μg/mL hydrocortisone, 10 μg/mL insulin, 100 ng/mL cholera toxin, and 50 μg/mL P/S. DLD-1 cells were cultured in RPMI-1640 (Life Technologies) containing 10% FBS (Life Technologies) and 50 μg/mL P/S. *Mycoplasma* test for cell culture was done in a yearly basis using the MycoPlasma Detection Kit (biotool.com). Cells used in experiments were within 10 passages from thawing. Cell dissociation buffer Enzyme-free PBS-based (13151-014) was purchased from Life Technologies. When indicated, cells were treated with pervanadate for 15 minutes before harvest. Pervanadate was prepared by mixing 1 part of 3 mol/L H_2O_2 with 1 part of 100 mmol/L sodium orthovanadate in PBS, and incubated at 25°C for 15 minutes prior to use at a ratio of 1:500 into culture medium.

Antibodies, plasmids, and other materials

See Supplementary Material for source of reagents.

Immunoprecipitation and kinase assay

These experiments were performed as described in ref. 4. Src kinase assay is similar except that GST-Lats1 purified from HEK293A was used as substrate.

Immunohistochemistry and immunofluorescence staining

Human breast cancer tissue microarrays were purchased from US Biomax. Arrays were deparaffinized through graded ethanol solutions. After an antigen retrieval procedure of 30 minutes using target retrieval solution (DAKO), the sections were stained with specific antibodies using the avidin–biotin complex system (Vector Laboratory). 3,3′-Diaminobenzidine (DAB) was used as the substrate. Cell nuclei were counterstained with hematoxylin.

For immunofluorescence staining, cells were cultured on cover slips to appropriate density. Cells were fixed with 4% paraformaldehyde for 15 min and then permeabilized with 0.1% Triton X-100. After blocking in 3% BSA for 30 min, slides were incubated with first antibody diluted in 1% BSA for 1.5 hours. After washing with PBS, slides were incubated with Alexa Fluor 488 or 594 conjugated secondary antibodies (1:1,000) for 1.5 hours. The slides were then washed and mounted.

Soft agar colony formation assay

NIH-3T3 cells (5×10^4) or HeLa cells (2.5×10^3) were added to 1.5 mL of growth medium with 0.4% agarose and layered onto 2 mL of 0.75% agarose beds in 6-well plates. Cells were fed with 2 mL of growth medium every week for 3 weeks, after which colonies were stained, pictured, and counted with Image J.

Colony formation assay

NIH-3T3 cells were seeded in 6-well plates at a density of 5×10^5 cells per well and then infected with v-src and shYAP plasmids. After 24 hours, cells were replated into 6-cm dish and maintained in DMEM supplemented with 5% FBS for a week, and then 1% FBS for another 2 to 3 weeks until foci were evident. Cells were then fixed with 10% acetic acid and 10% methanol, and then colonies were stained with 1% Crystal violet and counted with Image J.

Luciferase assays

For luciferase assays, cells were transfected with the reporter, CMV-β-gal, and indicated plasmids. 36 hours after transfection, cells were lysed and luciferase activity was assayed using the Luciferase Assay System (Promega) following the manufacturer's instructions. All luciferase activities were normalized to β-galactosidase activity.

Xenograft tumorigenesis model

All animal study protocols were approved by the Zhejiang University Animal Care and Use Committee. Nude mice (nu/nu, male 6- to 8-week-old) were injected subcutaneously with 5×10^5 infected NIH-3T3 cells. Around 3 to 4 weeks after injection, tumors were dissected, pictured, and weighted.

Structural modeling of Last1 kinase domain

The crystal structure of AKT in complex with its substrate peptide from glycogen synthase kinase-3 beta was obtained from Protein Data Bank (PDB ID: 3CQU). The structure of the Last1 kinase domain was modeled using WinCoot. The structure refinement was done using CCP4. This was performed by the refinement and structure idealization options in the software to idealize the protein geometry. Related figures were made by PyMOL.

Additional experimental details are available in the Supplementary Material.

Results

Lats1 is tyrosine-phosphorylated upon cell adhesion

We have previously reported that the Hippo pathway is regulated by cell adhesion, which inhibits Lats activity and thus activates YAP (26). Conversely, cell detachment activates Lats and thus inhibits YAP and induces anoikis. Indeed, we found that endogenous Lats1 immunoprecipitated from cells cultured in adhesion was repressed as indicated by diminished activity to phosphorylate YAP *in vitro* (Fig. 1A). The inhibition of Lats upon

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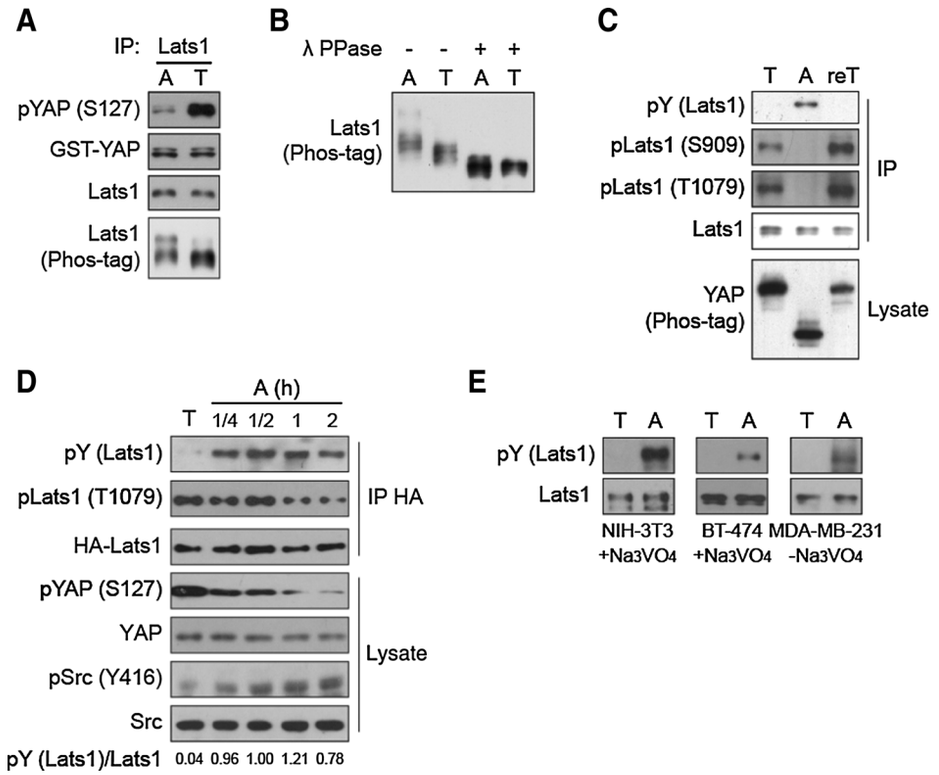

Figure 1.
Cell–matrix adhesion induces tyrosine phosphorylation of Lats1. **A,** Cell adhesion induces an electrophoretic upshift of Lats1. Hela cells were plated onto fibronectin-coated dishes for 3 hours (A) or further trypsinized (T). Immunoprecipitated Lats1 was used to phosphorylate GST-YAP *in vitro*. **B,** Lats1 mobility shift is due to phosphorylation. Cells were treated and harvested as that in **A**. Aliquots of the lysates were treated with lambda protein phosphatase before resolving on Phos-tag gels. **C,** Cell adhesion to matrix induces Lats1 tyrosine phosphorylation. Cells were trypsinized (T), attached for 3 hours (A), and then trypsinized again (reT). Lats1 was immunoprecipitated and detected by Western blotting. **D,** Cells dissociated by an enzyme-free method exhibit Lats1 tyrosine phosphorylation upon adhesion. Experiments were similar to those in **C**, except that an enzyme-free cell dissociation buffer was used. The ratio of pY (Lats1) to Lats1 was calculated from quantified band intensities. **E,** Lats1 tyrosine phosphorylation in cell adhesion condition is cell type independent. Cells were treated with pervanadate for 15 minutes before harvest when indicated. Data are representative of at least duplicate experiments.

cell adhesion is accompanied by repression of the activation loop and the hydrophobic motif phosphorylations, which are important for Lats activity (26). Surprisingly, we observed that adhesion induced an electrophoretic upshift of Lats1 on Phos-tag gel, which retards phosphorylated proteins, suggesting enhanced Lats1 phosphorylation (Fig. 1A). Furthermore, this upshift of Lats1 was eliminated by lambda protein phosphatase treatment of cell lysates (Fig. 1B). These results indicate that Lats1 inhibition by cell adhesion correlates with Lats1 phosphorylation. To identify the elusive phosphorylation sites, we made 29 serine/threonine to alanine mutations on residues documented in the PhosphoSite database. However, none of these mutations eliminated the upshift of Lats1 upon cell adhesion (data not shown). Tyrosine phosphorylation is another key posttranslational protein modification mediating cellular response to various signals such as cell adhesion. Interestingly, we found that cell adhesion induced tyrosine phosphorylation of endogenous Lats1, which correlated with repressed Lats1 activation loop (S909) and hydrophobic motif (T1079) phosphorylations, as well as YAP phosphorylation (Fig. 1C). Additionally, phospho-tyrosine could be detected at a molecular weight similar to the adhesion-induced upper band of Lats1 on Phos-tag gel (Supplementary Fig. S1A). To further rule out any potential artifact caused by trypsin digestion, cells were dissociated using an enzyme-free buffer followed by recovery in suspension for 4 hours before put in adhesion again. In this way, we also observed quick dephosphorylation of YAP accompanied by tyrosine phosphorylation of Lats1 (Fig. 1D). Moreover, we found that in additional cell lines tested including mouse fibroblast cell line NIH-3T3, breast cancer cell lines BT-474 and MDA-MB-231, and embryonic kidney cell line HEK293T, Lats1 tyrosine phosphorylation could be detected upon cell adhesion, which was enhanced by treatment of cells with tyrosine phosphatase inhibitor pervanadate before harvest (Fig. 1E and Supplementary Fig. S1B). These data indicate that cell adhesion induces Lats1 tyrosine phosphorylation in a cell-type–independent manner.

Src phosphorylates Lats1 upon cell adhesion

To identify the tyrosine kinase that phosphorylates Lats1, we screened a library of plasmids encoding 72 out of the 90 protein tyrosine kinases in the human kinome. We found that coexpression of Src clearly induced an electrophoretic upshift of Lats1 (Supplementary Fig. S2A). Indeed, Src but not the kinase inactive mutant clearly induced tyrosine phosphorylation of Lats1, while the phosphorylation of Lats2 was much weaker (Fig. 2A). Notably, the expression of ectopic Src was controlled at a level comparable to endogenous Src in breast cancer cell lines to avoid artifacts caused by excessive overexpression (Supplementary Fig. S2B). Although other Src family kinases (SFK) did not cause obvious shift of Lats1 in the screen, we found Fyn and Yes1 could also phosphorylate Lats1 albeit at lower efficiency (Supplementary Fig. S2C). To determine whether Src directly phosphorylates Lats1, we carried out *in vitro* kinase assay. Indeed, Src but not the kinase inactive mutant phosphorylates purified Lats1 *in vitro* as indicated by an obvious electrophoretic upshift and by immunoblotting with a phospho-tyrosine specific antibody (Fig. 2B).

It was well known that Src was activated by cell adhesion to matrix (27), which was confirmed in our experiments by

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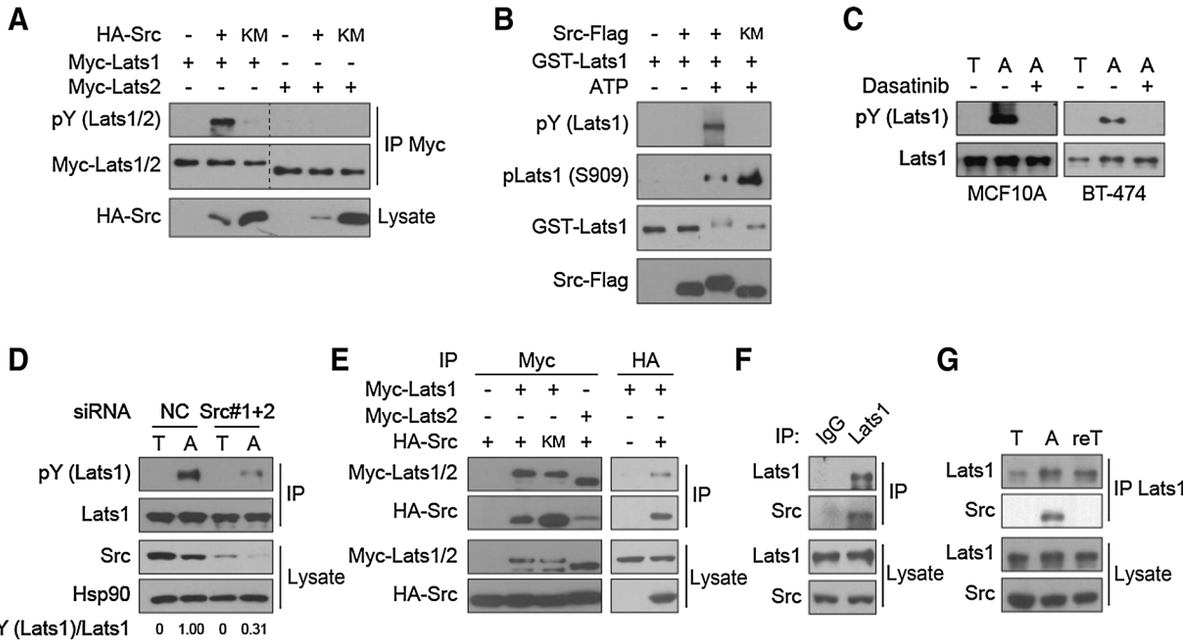

Figure 2.

Src phosphorylates Lats1. **A,** Src induces Lats1 tyrosine phosphorylation. HEK293T cells were transfected and Lats was immunoprecipitated. KM, the kinase inactive form of Src. **B,** Src phosphorylates Lats1 *in vitro*. Src expressed in HEK293T cells was immunoprecipitated with anti-Flag antibody and then subjected to *in vitro* kinase assay using GST-Lats1 as substrate. **C,** Src inhibitor blocks Lats1 tyrosine phosphorylation upon cell adhesion. Cells were trypsinized and attached again with or without 0.5 μmol/L dasatinib for 2 hours. Cells were treated with pervanadate for 15 minutes before harvest. **D,** Src knockdown inhibits Lats1 tyrosine phosphorylation upon cell adhesion. BT-474 cells transfected with negative control or Src siRNAs were trypsinized or further put in adhesion. Cells were treated with pervanadate for 15 minutes before harvest. Lats1 was immunoprecipitated. The ratio of pY (Lats1) to Lats1 was calculated from quantified band intensities. **E,** Src coimmunoprecipitates with Lats. HEK293T cells were transfected and lysates were immunoprecipitated as indicated. **F,** Endogenous Lats1 coimmunoprecipitates with Src. MCF10A cell lysates were immunoprecipitated with anti-Lats1 antibody and examined by Western blots. **G,** Cell adhesion to matrix promotes Src–Lats1 interaction. MCF10A cells were trypsinized or further put in adhesion and then trypsinized again. Lats1 was immunoprecipitated. Data are representative of at least duplicate experiments.

examining autophosphorylation of Src tyrosine 416 (Y416; Fig. 1D). To determine whether Src plays a role in Lats1 tyrosine phosphorylation upon cell adhesion, we examined the effect of Src inhibition. We found that among several examined Src inhibitors, dasatinib most significantly inhibited Src activity (Supplementary Fig. S2D). Indeed, dasatinib blocked Lats1 tyrosine phosphorylation upon cell adhesion (Fig. 2C). Furthermore, knockdown of Src by siRNAs also strongly inhibits Lats1 tyrosine phosphorylation induced by cell adhesion (Fig. 2D). We further examined whether Src could physically interact with Lats1. By coimmunoprecipitation, we found that Src wild-type protein could interact with Lats1, and the kinase inactive Src exhibited even higher interaction (Fig. 2E). Lats2 exhibited weaker interaction with Src (Fig. 2E), which might be underlying the weaker phosphorylation of Lats2 by Src. In addition, we could also detect coimmunoprecipitation of endogenous Src and Lats1 (Fig. 2F), which was enhanced by cell adhesion and inhibited by trypsinization (Fig. 2G). These data suggest that cell adhesion promotes Lats1 tyrosine phosphorylation through enhanced Src–Lats1 interaction. The above data demonstrate that Src is the major kinase phosphorylating Lats1 upon cell adhesion.

Src inhibits Lats1 activity

We next determined whether phosphorylation by Src inactivates Lats1. Indeed, while Src induced tyrosine phosphorylation on Lats1, it strongly inhibited phosphorylation of both S909 and T1079 residues (Fig. 3A). However, Lats2 phosphorylation on the same sites was not inhibited (Fig. 3A), suggesting that Src specifically inhibits Lats1. Furthermore, in an *in vitro* Src kinase activity assay, we found that Lats1 autophosphorylated its own activation loop, which was inhibited in the presence of Src (Fig. 2B). In addition, we established a Lats1 activity assay, in which Lats1 but not the kinase inactive KR mutant strongly phosphorylated YAP *in vitro* (Fig. 3B). Interestingly, coexpression with Src clearly inhibited Lats1 activity toward YAP (Fig. 3B). In contrast, Lats2 activity was not inhibited by Src (Fig. 3B). We next determined whether Src plays a role in inhibiting Lats1 activity upon cell adhesion. Indeed, dasatinib treatment or Src knockdown largely rescued Lats1 phosphorylation on the hydrophobic motif upon cell adhesion (Fig. 3C and D). C-terminal Src kinase (CSK) plays a key role in Src inhibition by phosphorylating Src on the C-terminal and thus inducing intramolecular interactions (28). Interestingly, knockdown of endogenous CSK not only enhanced Src autophosphorylation but also enhanced Lats1 tyrosine phosphorylation (Fig. 3E and Supplementary Fig. S3A). At the same time, Lats1 hydrophobic motif phosphorylation and YAP phosphorylation were inhibited (Fig. 3E). Consistently, in newly attached cells, overexpression of CSK inhibited Lats1 tyrosine phosphorylation and promoted Lats1 hydrophobic motif as well as YAP phosphorylation (Supplementary Fig. S3B). These findings demonstrate that Src inhibits Lats1 activity through tyrosine phosphorylation of Lats1.

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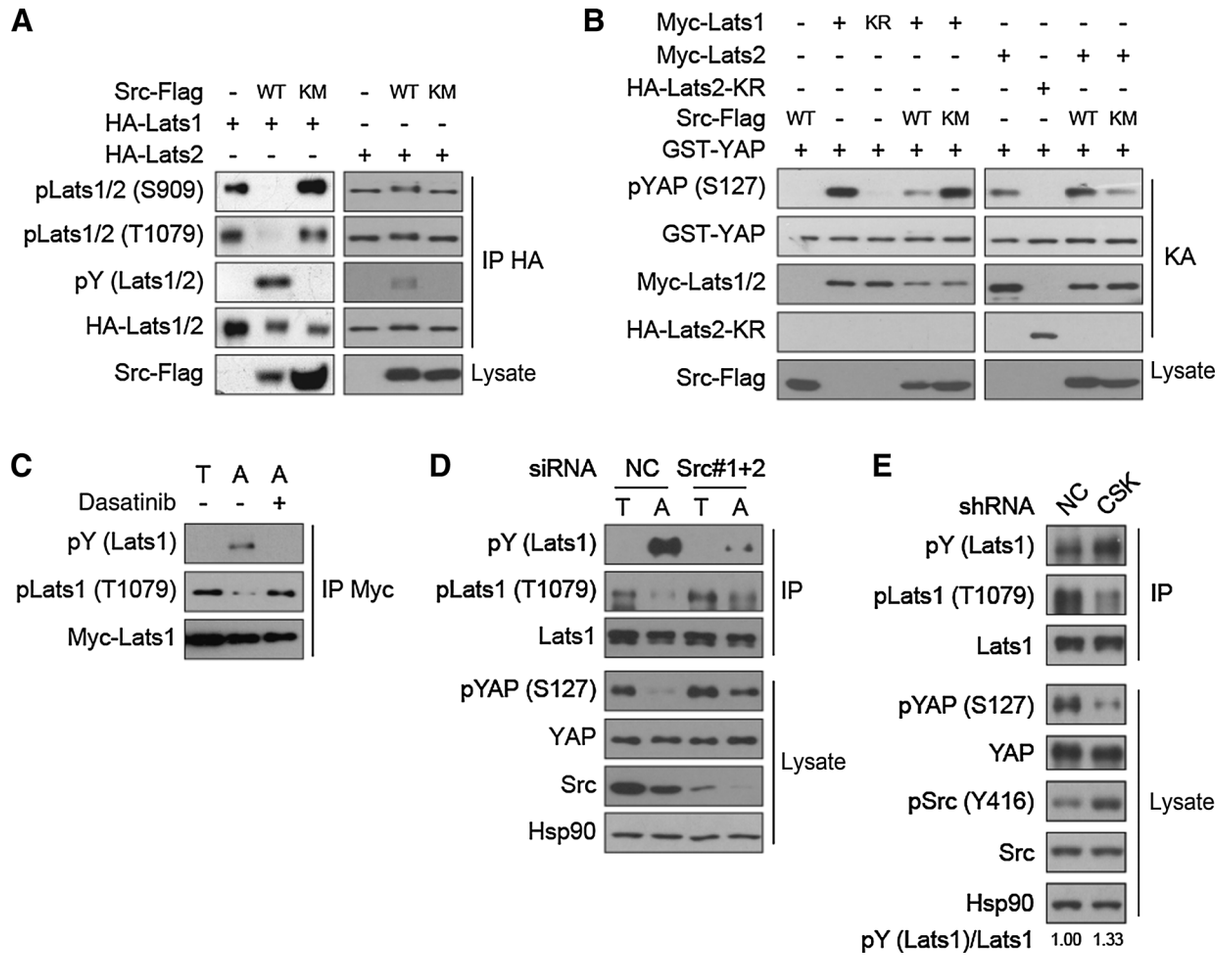

Figure 3.

Src inhibits Lats1 activity. **A,** Src inhibits Lats1 phosphorylations on the activation loop and the hydrophobic motif. HEK293T cells were transfected and lysates were immunoprecipitated as indicated. **B,** Src inhibits Lats1 kinase activity. HEK293T cells were cotransfected with Src and Lats as indicated. Lats was then immunoprecipitated with anti-Myc or HA antibodies and then subjected to *in vitro* kinase assay. **C,** Src inhibitor rescues Lats1 T1079 phosphorylation upon cell adhesion. Myc-Lats1–transfected HEK293T cells were trypsinized or further put in adhesion with or without 0.1 μmol/L dasatinib for 2 hours. Lats1 was immunoprecipitated. **D,** Src knockdown rescues Lats1 inhibition upon cell adhesion. BT-474 cells transfected with negative control or Src siRNAs were trypsinized or further put in adhesion. Cells were treated with pervanadate for 15 minutes before harvest. Lats1 was immunoprecipitated. **E,** Knockdown of CSK reduces Lats1 activity. MCF10A cells were infected with negative control or shRNA targeting CSK, and Lats1 was immunoprecipitated from the lysates. Cells were treated with pervanadate for 15 minutes before harvest. The ratio of pY (Lats1) to Lats1 was calculated from quantified band intensities. Data are representative of at least duplicate experiments.

Src phosphorylates Lats1 on multiple residues

To further understand how Src regulates Lats1, we mapped Lats1 tyrosine residues phosphorylated by Src. Lats1 was truncated into several fragments guided by the domain organization (Fig. 4A). When coexpressed with Src, the N-terminal fragments (amino acid 1–300, 301–552) of Lats1 were not phosphorylated and the Mob-binding region (635–704) had weaker phosphorylation. However, other fragments were comparably phosphorylated (Supplementary Fig. S4A and S4B). This suggests that Src could phosphorylate Lats1 on multiple residues. We then dissected each individual fragment by mutating tyrosines to phenylalanines. In this way, we found that Y573 is the major Src phosphorylation site in the fragment 1–634 (Fig. 4B) and Y916 is the major site in the fragment 705–1130 (Fig. 4C). The fragment 635–704 contains only one tyrosine residue Y692, which should account for phosphorylation of this fragment by Src. However, individually mutating any of these residues on the full-length Lats1 did not inhibit tyrosine phosphorylation, and combinational mutation of Y573/692/916 causes only a slight decrease of tyrosine phosphorylation (Fig. 4D). Furthermore, mutation of Y573/692/916 did not eliminate the adhesion-induced electrophoretic mobility shift of Lats1 on Phos-tag gel, although there was a slight downshift (Supplementary Fig. S4C). These data indicate that Src could phosphorylate additional tyrosine residues of Lats1. We therefore determined whether the identified sites could play a functional role in the regulation of Lats1 activity. Interestingly, mutation of Y692 or Y916 but not Y573 to glutamic acid, which mimics the charge of the phosphate group, represses Lats1 phosphorylation on the hydrophobic motif and inhibits Lats1 kinase activity (Fig. 4E). Furthermore, the Y692E and Y916E mutants of Lats1 also lost

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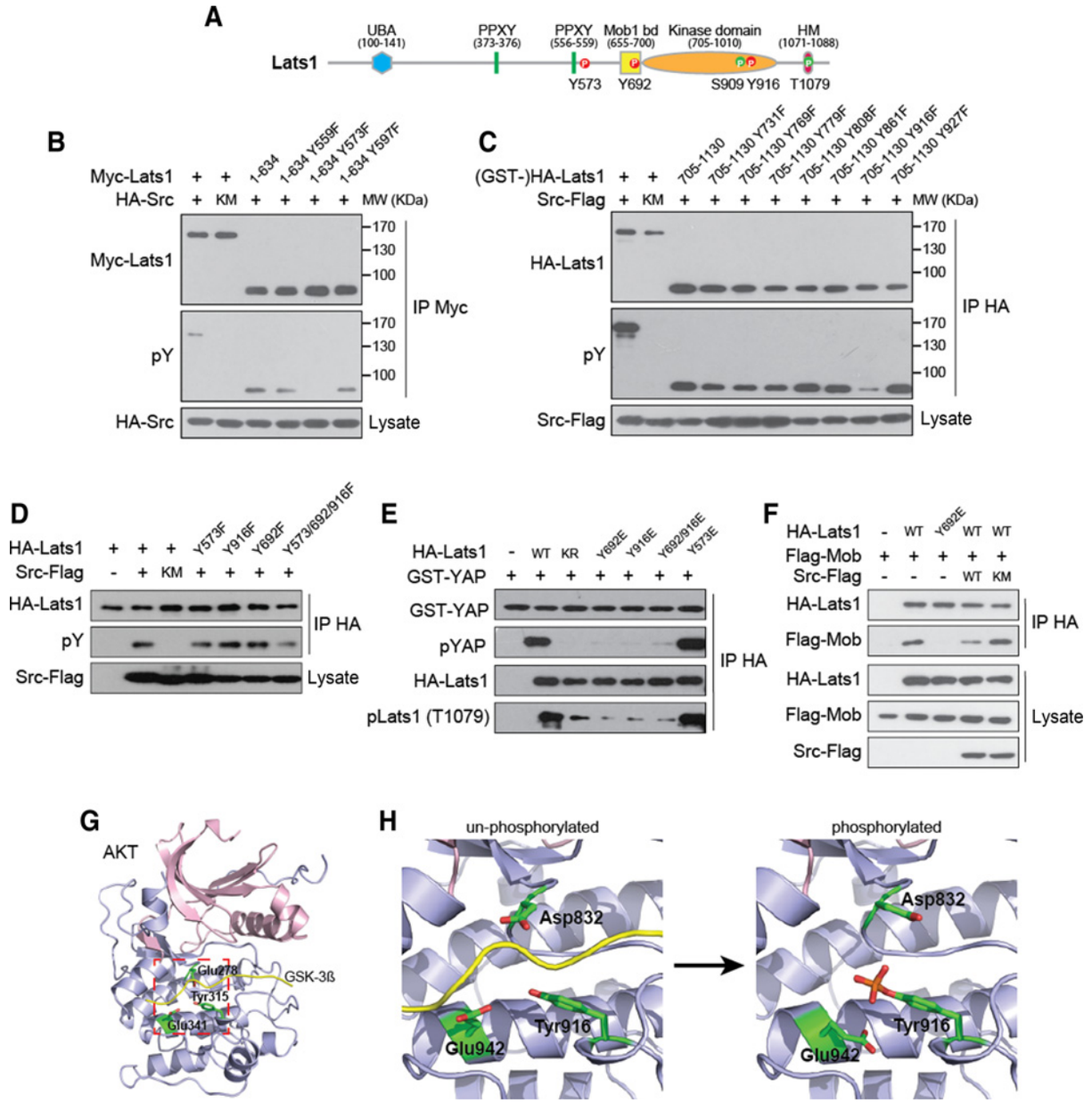

Figure 4.

Src phosphorylates Lats1 on multiple residues. **A,** Lats1 domain organization. Domains and phosphorylation sites of Lats1 were illustrated in scale. **B,** Y573 is the major phosphorylation site on Lats1 N-terminal 1-634 fragment. Lats1 was immunoprecipitated from HEK293A cells transfected as indicated. **C,** Y916 is the major phosphorylation site on Lats1 C-terminal 705-1130 fragment. **D,** Src phosphorylates Lats1 on multiple residues. Experiments were similar to those in **B**. **E,** Phosphomimetic mutations of Lats1 on Y692 and Y916 inhibit Lats1 kinase activity. Wild-type and mutant Lats1 proteins were immunoprecipitated from HEK293A cells and subjected to *in vitro* kinase assay. **F,** Phosphorylation of Y692 by Src inhibits Lats1 interaction with Mob. HEK293T cells were transfected and Lats1 was immunoprecipitated as indicated. **G,** The crystal structure of AKT in complex with its substrate peptide from GSK-3β. AKT is shown in cartoon, with the N-lobe in light pink and the C-lobe in light blue. The three residues are shown in stick representations. The substrate peptide is in yellow color. **H,** Zoom-in views of the model of Last1 kinase domain with Y916 unphosphorylated (left) or phosphorylated (right) based on the crystal structure of AKT. Data are representative of at least triplicate experiments.

the ability to inhibit YAP transcriptional activity (Supplementary Fig. S4D). These data suggest that Src could inhibit Lats1 activity through phosphorylation of Lats1 Y692 and Y916.

Y692 is in the Mob binding domain of Lats1. Accordingly, we found that Src inhibited the interaction between Lats1 and Mob in a kinase activity–dependent manner (Fig. 4F). Furthermore, the Y692E mutant of Lats1 lost interaction with Mob (Fig. 4F). Therefore, Y692 phosphorylation by Src may inhibit Lats1 activity through suppressing the Lats1–Mob interaction. Y916 is in the kinase domain of Lats1, but mutation of Y916 to phenylalanine

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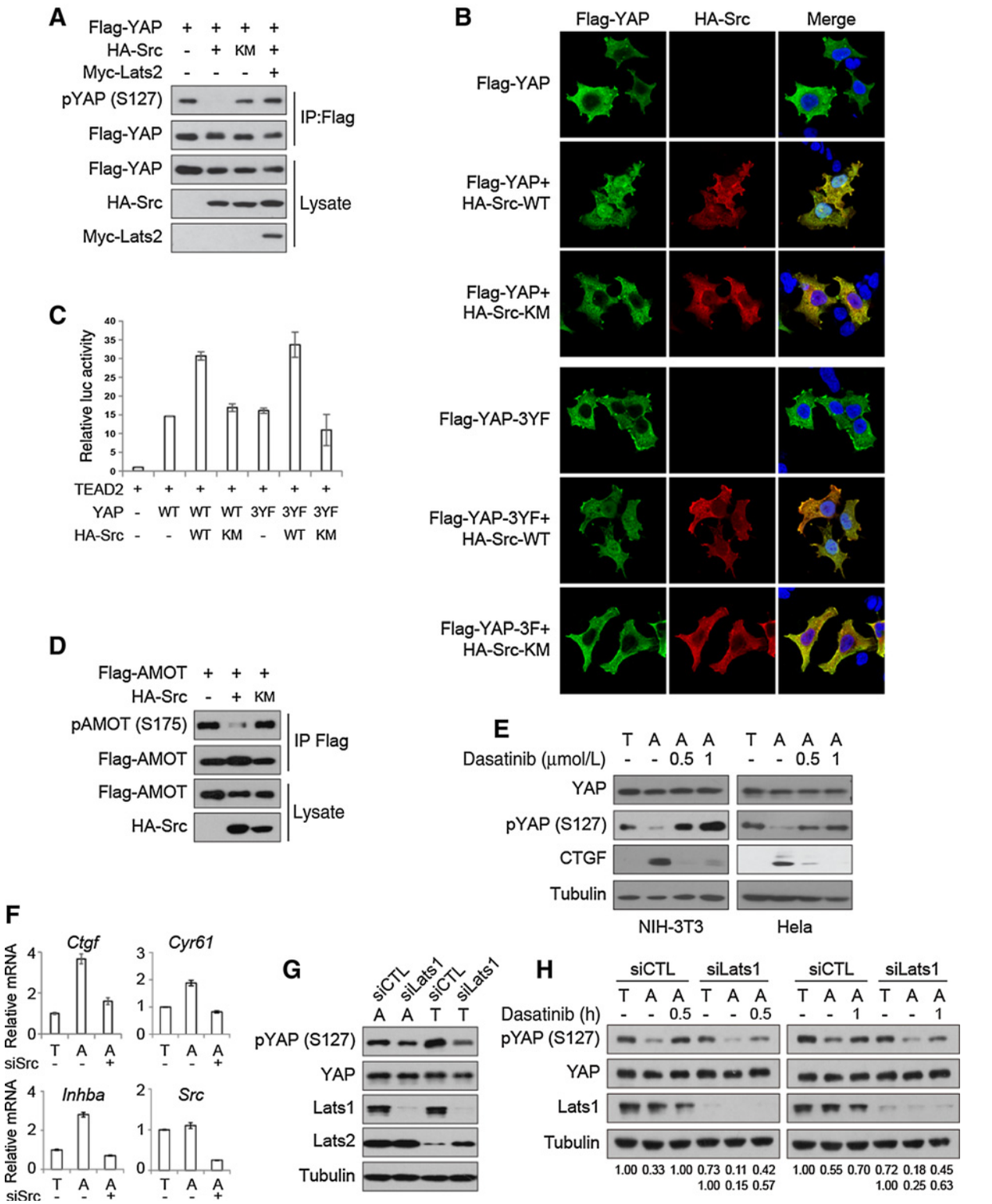

Figure 5.

Src is involved in YAP activation by cell adhesion. **A,** Src inhibits YAP phosphorylation. HEK293T cells were transfected and YAP was immunoprecipitated. **B,** Src promotes YAP nuclear localization. HEK293T cells were transfected as indicated. YAP and Src were stained with anti-Flag and anti-HA antibodies, respectively. **C,** Src promotes YAP transcriptional activity. HEK293T cells were transfected with CTGF-luc reporter and other plasmids as indicated for luciferase assay. **D,** Src inhibits phosphorylation of AMOT. HEK293T cells were transfected as indicated and AMOT was immunoprecipitated. (*Continued on the following page.*)

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does not inhibit Lats1 activity (Supplementary Fig. S4E), suggesting that Y916 is not structurally required and supporting a specific regulatory role for phosphorylation on this residue. Although the crystal structure of Lats1 is not available, the Y916 residue is highly conserved in the kinase domain of other AGC family kinases with solved structures, such as AKT (Supplementary Fig. S4F). The crystal structure of AKT in complex with its substrate peptide from glycogen synthase kinase-3 beta (GSK-3β; PDB ID: 3CQU; ref. 29) reveals that Y315, E278, and E341 of AKT form part of the substrate binding site (Fig. 4G). While Y315 does not directly contact the substrate peptide, E278 and E341 are in direct contact with the −5 position arginine residue. The three residues are highly conserved across the AGC family, and the corresponding residues in Last1 are Y916, D832, and E942 (Supplementary Fig. S4F). Based on the crystal structure of AKT, we modeled Last1 with Y916 unphosphorylated or phosphorylated (Fig. 4H). The models indicated that addition of a phosphate moiety on Y916 might sterically induce side-chain conformational changes on D832 and E942, thus together disrupting the substrate-binding site. Taken together, Src phosphorylates Lats1 on multiple residues and phosphorylation of Y692 and Y916 leads to Lats1 inhibition.

Src inhibition of Lats1 is involved in YAP activation upon cell adhesion

YAP is the major effector phosphorylated by Lats1 in the Hippo pathway. We thus examined the effect of Src on YAP phosphorylation. Indeed, YAP phosphorylation on S127, a major Lats target site, was strongly repressed by Src in a kinase-dependent manner (Fig. 5A). Phosphorylation by Lats destabilizes TAZ, the YAP paralog (8). Consistently, coexpression of Src elevated the TAZ protein level (Supplementary Fig. S5A), suggesting repressed TAZ phosphorylation. Phosphorylation of YAP on S127 promotes YAP cytoplasmic retention (4). Consistently, we observed a clear translocation of YAP to cell nuclei induced by Src but not the kinase inactive mutant (Fig. 5B). Similarly, by subcellular fractionation, we found that Src also promoted nuclear localization of TAZ (Supplementary Fig. S5B). Furthermore, using a reporter driven by the promoter of CTGF, a well-characterized YAP target gene (10), we found that YAP activity was also enhanced by Src in a kinase-dependent manner (Fig. 5C). These data demonstrate that Src inhibits YAP phosphorylation by the Hippo pathway and thus promotes YAP activity.

However, it was previously reported that YAP1 could be directly phosphorylated by Src on three tyrosine residues (30), which are all conserved in the YAP2 isoform (a splicing variant of YAP with one more WW domain compared with YAP1). We therefore asked whether tyrosine phosphorylation of YAP could alternatively explain repression of YAP S127 phosphorylation by Src. Several pieces of evidence argue against the possibility. First, mutation of all three tyrosine residues (Y375/391/428) on YAP2 (used throughout the study unless specified) did not affect YAP nuclear translocation or activation induced by Src (Fig. 5B and C). Second, coexpression of Lats2, which is resistant to Src, rescued YAP S127 phosphorylation and TAZ protein level in the presence of Src (Fig. 5A and Supplementary Fig. S5A). Third, phosphorylation of AMOT, another substrate of Lats1, was also inhibited by Src (Fig. 5D). Fourth, using two different phospho-tyrosine–specific antibodies, we could not detect YAP2 or YAP1 tyrosine phosphorylation by Src (Supplementary Fig. S5C and S5D). Fifth, we could not observe direct phosphorylation of YAP by Src *in vitro* using phospho-tyrosine–specific antibody as the detection method (Supplementary Fig. S5E). These data support that Src inhibits YAP phosphorylation on S127 through inhibition of the respective kinase Lats1.

We further examined whether Src plays a role in YAP activation upon cell adhesion. We found that dasatinib not only rescued YAP phosphorylation on S127, but also inhibited induction of endogenous CTGF (Fig. 5E) as well as other YAP target genes (Supplementary Fig. S5F). Furthermore, knockdown of Src with siRNAs largely rescued YAP phosphorylation (Fig. 3D) and inhibited YAP target gene expression in response to cell adhesion (Fig. 5F and Supplementary Fig. S5G). Both Lats1 and Lats2 activities are regulated by cell adhesion (26). We thus determined the contribution of Lats1 phosphorylation by Src in the regulation of YAP by cell adhesion. Interestingly, in some cells such as the DLD-1 colorectal cancer cell line, knockdown of Lats1 significantly reduced detachment-induced YAP phosphorylation (Fig. 5G). However, in some other cell lines such as HEK293T, HeLa, and MCF10A, knockdown of Lats1 reduced but did not block YAP phosphorylation induced by cell detachment (Fig. 5H). Nevertheless, when HEK293T cells were attached for a short time, Lats1 knockdown rendered dasatinib less effective in rescuing YAP phosphorylation (Fig. 5H). However, when cells were attached for a longer time, rescue of YAP phosphorylation by dasatinib was less complete in control cells and the difference between control and Lats1 knockdown cells was obscure (Fig. 5H). This result suggests that Lats1 inhibition by Src, which is activated at focal adhesions, may play a more prominent role at the early stage of cell adhesion. But signal from cytoskeleton remodeling likely ensues and further inhibits Lats2 through other mechanisms. Noteworthy, in DLD-1, the Lats2 protein level was clearly repressed by cell detachment (Fig. 5H), which may result in a sensitized background for YAP regulation by Lats1.

It was previously reported that Src might inhibit Lats1 through activation of the PI3K pathway (31). However, we found that while LY294002 and wortmannin, two PI3K inhibitors, strongly inhibited phosphorylation of classical PI3K downstream target AKT, they could not rescue Lats1 or YAP phosphorylation (Supplementary Fig. S5H). Nevertheless, dasatinib clearly rescued Lats1 and YAP phosphorylation, although its repression of AKT phosphorylation was less potent compared with LY294002 and wortmannin (Supplementary Fig. S5H). These data suggest that Src inhibits the Hippo pathway upon cell adhesion mainly through a PI3K-independent manner.

(*Continued.*) **E,** Src inhibitor represses YAP activation upon cell adhesion. Cells were trypsinized or further put in adhesion with or without dasatinib for 2 hours. **F,** Knockdown of Src reduces YAP target gene expression upon cell adhesion. BT-474 cells were transfected with control or Src siRNAs. Gene expression levels were determined by quantitative PCR. **G,** Knockdown of Lats1 inhibits YAP phosphorylation induced by cell detachment in DLD-1 cells. Cells were transfected with control siRNA or a mixture of two Lats1 siRNAs. Cells were put in adhesion or trypsinized and sat in suspension for 1 hour. Cell lysates were examined by Western blotting. **H,** Src contributes to the regulation of YAP at the early phase of cell adhesion. HEK293T cells were transfected with siRNAs and trypsinized or put in adhesion with or without 0.1 μmol/L dasatinib for the indicated time. Western blots were quantified and relative ratios of phospho-YAP to total YAP are indicated at the bottom. For the siLats1 samples, ratios with the trypsinized sample as unit one are also indicated in a separate line. Data are means ± SD from three technical repeats and are representative of at least triplicate experiments.

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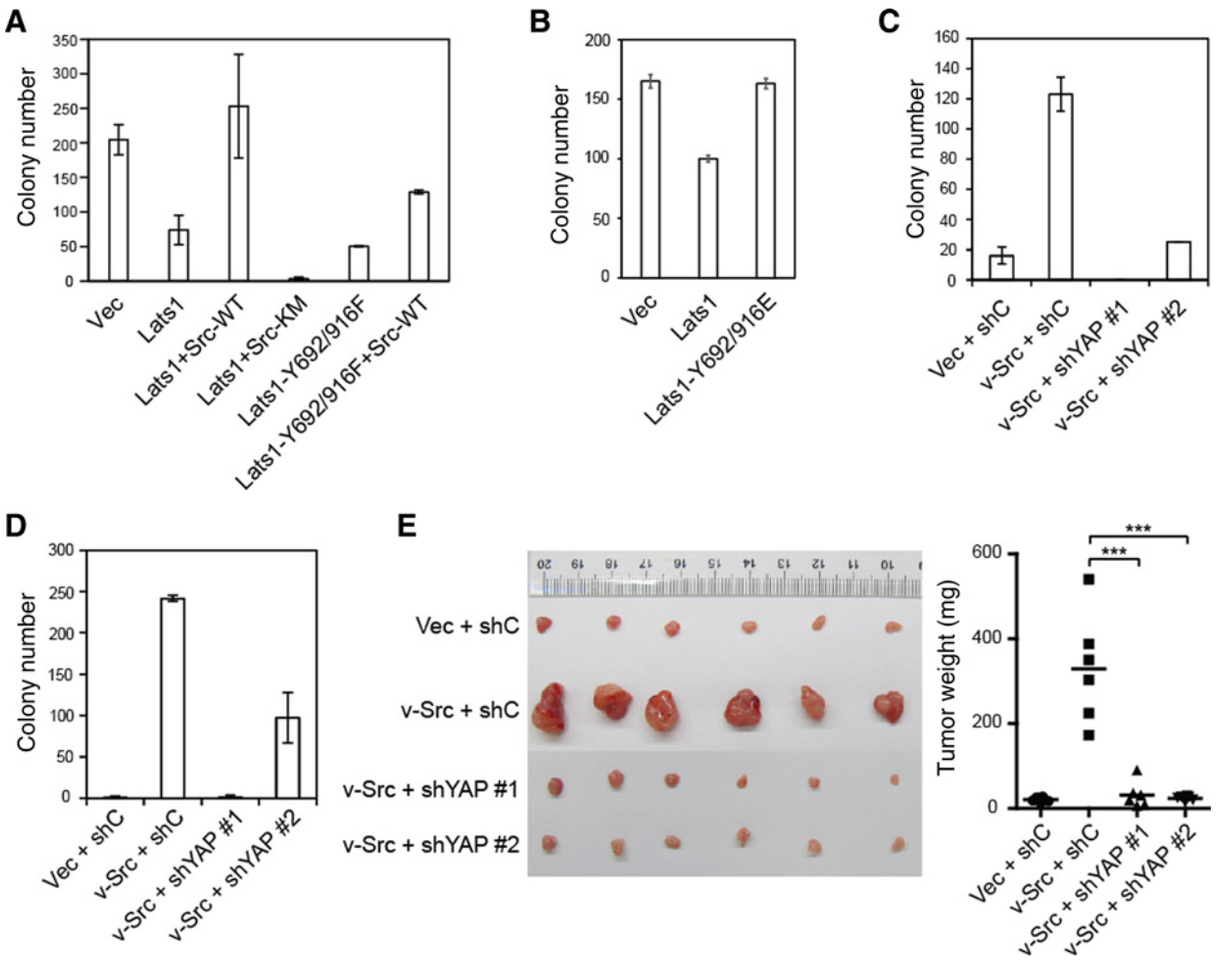

Figure 6.

Src promotes transformation and tumorigenesis through the Hippo pathway. **A,** Src represses the tumor suppressor activity of Lats1. HeLa cells were infected with Lats1 and Src wild-type or mutants and then cultured in soft agar. Colonies were quantified after 3 weeks. **B,** Phosphomimetic mutation of Lats1 inhibits its tumor suppressor activity. Experiments were similar to those in **A**. **C,** YAP is required for v-Src-induced anchorage-independent growth. NIH-3T3 cells were infected with v-Src and shRNAs against YAP as indicated. Cells were then cultured in soft agar. Colonies were stained and quantified after 3 weeks. **D,** YAP is required for v-Src-induced evasion of contact inhibition. NIH-3T3 cells, the same as those in **C**, were subjected to colony formation for 4 weeks on tissue culture plates. **E,** YAP is required for v-Src-induced tumorigenesis. Nude mice were injected subcutaneously on the two flanks, with cells the same as those in **C**. Tumors were dissected after 3 weeks. *P* value was calculated by Student *t* test. Data are means ± SD from technical duplicates and are representative of at least duplicate experiments.

The Hippo pathway is downstream of Src in tumorigenesis

Src is a proto-oncogene promoting cellular transformation and tumorigenesis. Since Src activates YAP through inhibition of Lats1, we asked whether the Hippo pathway plays a role in oncogenic transformation and tumorigenesis downstream of Src. In HeLa cells, Lats1 inhibited anchorage-independent growth, thus reduced colony formation in soft agar (Fig. 6A). Coexpression of Src suppressed the activity of Lats1 and fully rescued colony formation. However, expression of inactive Src further inhibited colony formation possibly due to a dominant negative effect (Fig. 6A). While the Y692/916F mutant of Lats1 remained active in suppressing HeLa cell colony formation, coexpression of Src only partially rescued colony formation, indicating that the Y692/916F mutant is resistant to Src (Fig. 6A). Furthermore, the phosphomimetic mutant Y692/916E lost the ability to suppress HeLa cell colony formation (Fig. 6B). Thus, Src inhibits the tumor suppressive activity of the Hippo pathway kinase Lats1.

We further examined whether YAP is required for the oncogenic activity of Src. v-Src is an active form of Src isolated from Rous sarcoma virus encoded by the prototype oncogene *v-src* (32). v-Src–induced NIH-3T3 cell growth in soft agar is one of the oldest oncogenic transformation assays. We have previously shown that expression of active YAP could induce a similar phenotype in NIH-3T3 cells (33). Interestingly, when YAP was inhibited by shRNA knockdown (Supplementary Fig. S6A), the induction of anchorage-independent growth by v-Src was blocked (Fig. 6C). Src could also induce evasion of cell contact inhibition, thus cells grow on top of each other to form colonies. In this assay, the transforming potential of v-Src was also largely inhibited by knockdown of YAP (Fig. 6D). We further investigated the role of YAP in Src-induced tumorigenesis *in vivo*. Expression of v-Src

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clearly promoted tumor growth in nude mice (Fig. 6E). In consistent with observations *in vitro*, YAP phosphorylation levels in Src-induced tumors were lower compared with these in the smaller control tumors (Supplementary Fig. S6B). Strikingly, knockdown of YAP markedly blocked Src-induced tumor growth (Fig. 6E). These findings demonstrate that inhibition of Lats1 and activation of YAP play critical roles downstream of Src to promote tumorigenesis.

Elevated Src protein levels in human breast cancer correlate with YAP activation

We next investigated whether deregulation of Src could be a reason for YAP activation in human cancer. In the pool of total YAP protein, only dephosphorylated YAP could get into the cell nucleus to activate gene expression thus playing an oncogenic role. To accurately evaluate the level of dephosphorylated active YAP, we used a newly developed antibody specifically recognizing YAP when dephosphorylated on the S127 site. The specificity of this antibody was demonstrated by Western blots indicating anticipated responsiveness of the detected band to stimulus and elimination of the signal by YAP knockout (Fig. 7A). In addition, phosphorylation of recombinant YAP *in vitro* also eliminated detection of YAP by this active YAP-specific antibody (Fig. 7B). Thus, this antibody could faithfully reflect the level of dephosphorylated active YAP. Src activity was found activated by 4- to 20-fold in human breast cancer (34). We thus carried out immunohistochemical (IHC) staining for both Src and active YAP on a human breast cancer tissue microarray containing 70 cases. The

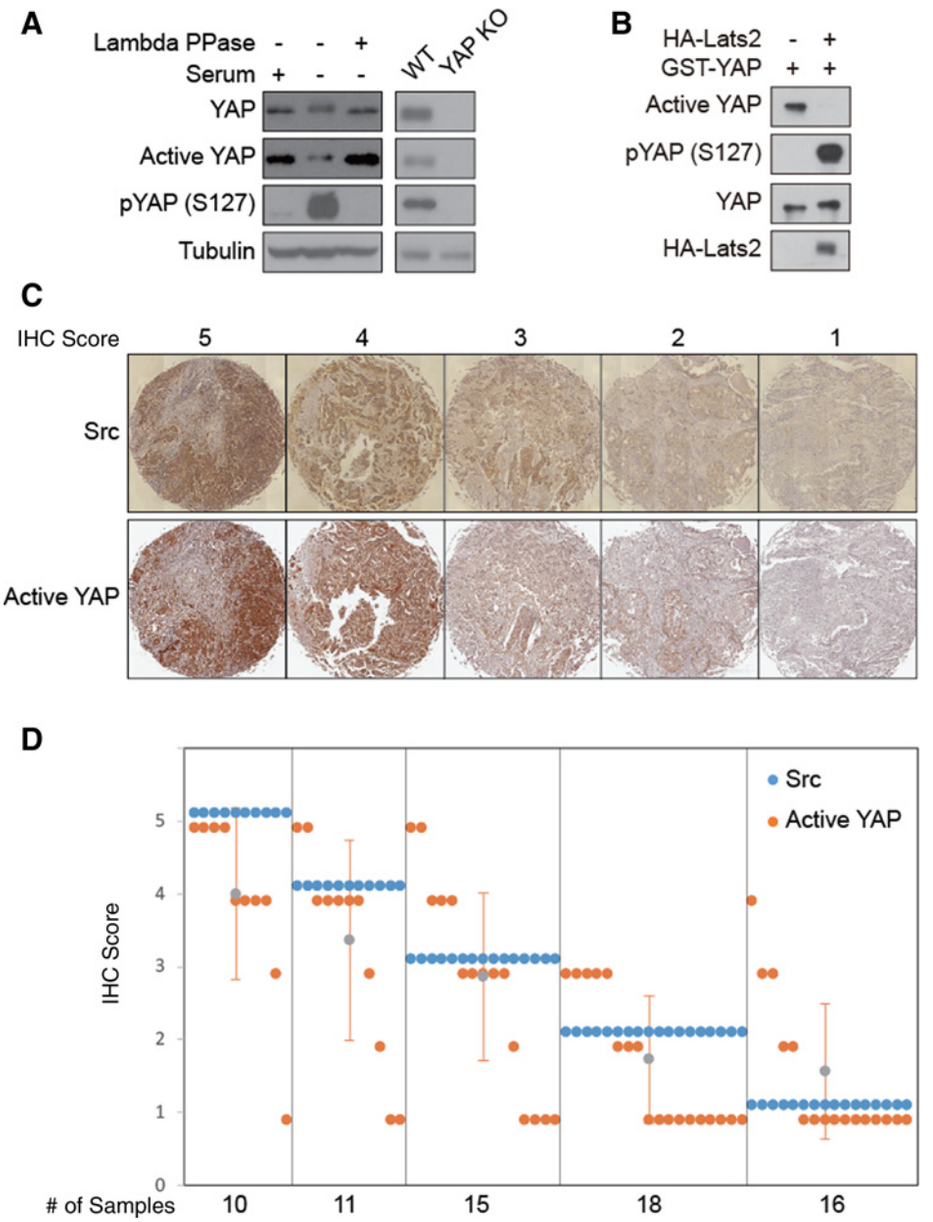

Figure 7.
YAP activation correlates with Src expression in breast cancer. **A,** Validation of an anti-active YAP antibody. HEK293T cells cultured in the presence or absence of serum and HEK293T cells with YAP knockout were lysed and analyzed by Western blotting. Lysates were treated with lambda protein phosphatase as indicated. **B,** Phosphorylation of recombinant YAP eliminates detection by the anti-active YAP antibody in Western blotting. Recombinant GST-YAP was phosphorylated by Lats2 *in vitro*. **C,** Representative pictures of IHC staining of Src and active YAP on human breast cancer tissue microarray scored from 1 to 5. **D,** Correlation of Src protein level with YAP activation. Cases on the tissue microarray were ranked by the IHC scores of Src. For each Src score group, the average score for active YAP was calculated (gray dot), means ± SD.

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level of Src and active YAP were graded 1 to 5 from low to high (Fig. 7C). When the samples were grouped by the score of Src staining, we found that groups express more Src (scores 4 and 5) also have higher level of active YAP, and many samples in groups with lower Src level (scores 1 and 2) have minimal active YAP (Fig. 7D). Although there are cases in which Src level does not match active YAP level indicating additional mechanisms for YAP deregulation, the general trend indicates that elevated Src expression correlates with the level of dephosphorylated active YAP in breast cancer, which further supports a role of Lats1 inhibition by Src in tumorigenesis.

Discussion

Mechanical stress is an important upstream signal of the Hippo pathway. For example, YAP is regulated by cell–cell adhesion, cell–matrix adhesion, matrix stiffness and geometry, shear flow and stretch (4, 26, 35, 36). Interestingly, tissue physical properties also play a profound role in tumorigenesis. It is known that tumor stroma is characterized by excessive stiffening of the extracellular matrix and reduction of tension represses the malignant behavior of mammary epithelial cells (37). Therefore, it is important to elucidate the mechanism of YAP regulation by mechanical signals such as cell adhesion. YAP regulation by mechanical signals involves modulation of Lats kinase activity (26, 38–41). In *Drosophila*, a couple of mechanisms have been suggested explaining Wts regulation by F-actin. One is that F-actin inhibits Wts interaction with NF2, which directs Wts to the plasma membrane for activation (42). The other is that cytoskeletal tension promotes Wts localization to adherens junctions in a Jub-dependent manner, which separates Wts from the upstream activating proteins (43). Whether similar mechanisms function in mammalian cells awaits further investigation. However, it was suggested that JNK phosphorylates LIMD1, a mammalian Jub homolog, under cell stretch condition, thus promotes its interaction with and inhibition of Lats (35). Despite these insights into Lats regulation by F-actin, it was unclear whether cell adhesion complexes, such as focal adhesions, directly regulate the Hippo pathway. Recently, Src has been implicated in Lats inhibition through activating PI3K and PDK1 under cell adhesion condition (31). However, how PDK1 regulates Lats activity is unclear and in our experimental conditions inhibition of PI3K or genetic knockout of PDK1 does not affect YAP phosphorylation (4). We propose that mechanical stress and cell adhesion regulate YAP through multiple mechanisms involving both actin cytoskeleton and Src at focal adhesion. Src directly phosphorylates Lats1 on tyrosine residues, resulting in attenuated Lats1 phosphorylation and activity. This mechanism is the first report of Lats1 activity being regulated by tyrosine phosphorylation and indicates a direct role of cell adhesion complex in Hippo pathway regulation.

YAP, as reflected by its name Yes-associated protein, was first cloned as an interacting protein of c-Yes, one of the SFKs (44). However, at the time of its cloning, YAP was not reported being phosphorylated by Yes. It was later that YAP was shown being tyrosine-phosphorylated by Src, which regulates YAP interaction with Runx2 and thus osteogenic gene expression. Furthermore, inflammatory signal through gp130 strongly promotes YAP protein level and nuclear localization in intestinal epithelium, which may also involve Src/Yes (45, 46). However, in the context of cell adhesion, we have difficulty to detect YAP being directly tyrosine phosphorylated by Src even with Src coexpression. Instead, we found that Src activates YAP through phosphorylation and inhibition of the canonical Hippo pathway kinase Lats1. This mechanism of YAP activation by Src is independent of the previously reported mechanism because mutation of the previously reported Src target sites on YAP does not block YAP nuclear translocation or activation induced by Src. Therefore, mechanisms underlying YAP regulation by Src could be context dependent.

Our finding that Src regulates Hippo pathway activity has important implication in cancer because while YAP activation was found in many human cancers, genetic alterations of YAP and the Hippo pathway are rare (2). The *Src* gene is a proto-oncogene identified in mammalian cells (32). Activation of Src is common in colon cancer, breast cancer, and is also found in prostate cancer, melanoma, ovarian cancer, gastric cancer, hepatocellular carcinoma, and several other types of cancers (47). Src makes important contribution to various aspects of cancer development including tumor cell proliferation and survival, metastasis, angiogenesis, and therapeutic resistance (47). Interestingly, the Hippo pathway and YAP have been demonstrated in a largely overlapping set of functions (2, 48). Therefore, Src activation is a potential mechanism causing YAP activation in human cancer. Indeed, we found that in human breast cancer, elevated Src protein level correlates with the level of dephosphorylated active YAP. Thus, our findings provide a new mechanism to explain the deregulation of the Hippo pathway in human cancer. Interestingly, this mechanism may also explain YAP deregulation in the tumor microenvironment. It was reported that through an unknown mechanism, dasatinib inhibits YAP nuclear localization, and interaction with TEAD in cancer-associated fibroblasts (CAF) thus regulates cytoskeleton and matrix stiffness (22). Our molecular model could explain the regulation of YAP by Src in CAFs and suggests a broader role of the mechanism in cancer.

While modulation of many signaling pathways such as PI3K–AKT signaling, Ras–MAPK signaling, STAT signaling and focal adhesion signaling, contributes to the functions of Src, our study suggests that Lats1 is a new effector of Src in cellular transformation and tumorigenesis. The cross-talk between Src and the Hippo pathway may also impact other aspects of cancer progression. Clinical trials of Src inhibitors in cancer have been extensively pursued with limited success as a single agent therapy, which could be due to lack of biomarkers and patient stratification, as well as additional complication by other molecular alterations (47). Therefore, the identification of the Hippo pathway downstream of Src suggests YAP activation as a potential biomarker for Src therapy and warrants further analysis of the impact of the Hippo pathway in Src therapy effectiveness. In this line, it was reported that YAP-dependent β-catenin active colon cancer cells are sensitive to the Src inhibitor dasatinib, but YAP-independent β-catenin inactive cells are insensitive (49). In addition, pharmacological targeting of YAP has also been actively investigated (50), which may provide novel agents for targeting Src-activated cancers.

In summary, we report tyrosine phosphorylation of Lats1 by Src as a novel mechanism of Hippo pathway regulation involved in cellular response to cell adhesion and in tumorigenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Ji, X. Cao, X. Dai, L. Xu, X. Guo, H. Yan, H. Zhang, C. Zhu, Q. Zhou, M. Tang, Z. Xia, S. Ye, T. Liang, X.-H. Feng, B. Zhao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Si, X. Dai, H. Zhao, B. Zhao
Writing, review, and/or revision of the manuscript: Y. Si, X. Cao, L. Li, Y.-S. Cong, X.-H. Feng, B. Zhao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.-S. Cong, T. Liang

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References

1. Pan D. The hippo signaling pathway in development and cancer. Dev Cell 2010;19:491–505.
2. Yu FX, Zhao B, Guan KL. Hippo pathway in organ size control, tissue homeostasis, and cancer. Cell 2015;163:811–28.
3. Lai ZC, Wei X, Shimizu T, Ramos E, Rohrbaugh M, Nikolaidis N, et al. Control of cell proliferation and apoptosis by mob as tumor suppressor, mats. Cell 2005;120:675–85.
4. Zhao B, Wei X, Li W, Udan RS, Yang Q, Kim J, et al. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes Dev 2007;21:2747–61.
5. Zhao B, Li L, Tumaneng K, Wang CY, Guan KL. A coordinated phosphorylation by Lats and CK1 regulates YAP stability through SCF(beta-TRCP). Genes Dev 2010;24:72–85.
6. Hao Y, Chun A, Cheung K, Rashidi B, Yang X. Tumor suppressor LATS1 is a negative regulator of oncogene YAP. J Biol Chem 2008;283:5496–509.
7. Lei QY, Zhang H, Zhao B, Zha ZY, Bai F, Pei XH, et al. TAZ promotes cell proliferation and epithelial-mesenchymal transition and is inhibited by the hippo pathway. Mol Cell Biol 2008;28:2426–36.
8. Liu CY, Zha ZY, Zhou X, Zhang H, Huang W, Zhao D, et al. The hippo tumor pathway promotes TAZ degradation by phosphorylating a phosphodegron and recruiting the SCF{beta}-TrCP E3 ligase. J Biol Chem 2010;285:37159–69.
9. Oka T, Mazack V, Sudol M. Mst2 and Lats kinases regulate apoptotic function of Yes kinase-associated protein (YAP). J Biol Chem 2008;283:27534–46.
10. Zhao B, Ye X, Yu J, Li L, Li W, Li S, et al. TEAD mediates YAP-dependent gene induction and growth control. Genes Dev 2008;22:1962–71.
11. Feng X, Degese MS, Iglesias-Bartolome R, Vaque JP, Molinolo AA, Rodrigues M, et al. Hippo-independent activation of YAP by the GNAQ uveal melanoma oncogene through a trio-regulated rho GTPase signaling circuitry. Cancer Cell 2014;25:831–45.
12. Yu FX, Luo J, Mo JS, Liu G, Kim YC, Meng Z, et al. Mutant Gq/11 promote uveal melanoma tumorigenesis by activating YAP. Cancer Cell 2014;25:822–30.
13. Zhang N, Bai H, David KK, Dong J, Zheng Y, Cai J, et al. The Merlin/NF2 tumor suppressor functions through the YAP oncoprotein to regulate tissue homeostasis in mammals. Dev Cell 2010;19:27–38.
14. Zender L, Spector MS, Xue W, Flemming P, Cordon-Cardo C, Silke J, et al. Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach. Cell 2006;125:1253–67.
15. Song Y, Li L, Ou Y, Gao Z, Li E, Li X, et al. Identification of genomic alterations in oesophageal squamous cell cancer. Nature 2014;509:91–5.
16. Fernandez LA, Northcott PA, Dalton J, Fraga C, Ellison D, Angers S, et al. YAP1 is amplified and up-regulated in hedgehog-associated medulloblastomas and mediates Sonic hedgehog-driven neural precursor proliferation. Genes Dev 2009;23:2729–41.
17. Overholtzer M, Zhang J, Smolen GA, Muir B, Li W, Sgroi DC, et al. Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. Proc Natl Acad Sci U S A 2006;103:12405–10.
18. Dong J, Feldmann G, Huang J, Wu S, Zhang N, Comerford SA, et al. Elucidation of a universal size-control mechanism in Drosophila and mammals. Cell 2007;130:1120–33.
19. Camargo FD, Gokhale S, Johnnidis JB, Fu D, Bell GW, Jaenisch R, et al. YAP1 increases organ size and expands undifferentiated progenitor cells. Curr Biol 2007;17:2054–60.
20. Shen S, Guo X, Yan H, Lu Y, Ji X, Li L, et al. A miR-130a-YAP positive feedback loop promotes organ size and tumorigenesis. Cell research 2015;25:997–1012.
21. Schlegelmilch K, Mohseni M, Kirak O, Pruszak J, Rodriguez JR, Zhou D, et al. Yap1 acts downstream of alpha-catenin to control epidermal proliferation. Cell 2011;144:782–95.
22. Calvo F, Ege N, Grande-Garcia A, Hooper S, Jenkins RP, Chaudhry SI, et al. Mechanotransduction and YAP-dependent matrix remodelling is required for the generation and maintenance of cancer-associated fibroblasts. Nat Cell Biol 2013;15:637–46.
23. Shao DD, Xue W, Krall EB, Bhutkar A, Piccioni F, Wang X, et al. KRAS and YAP1 converge to regulate EMT and tumor survival. Cell 2014;158:171–84.
24. Kapoor A, Yao W, Ying H, Hua S, Liewen A, Wang Q, et al. Yap1 activation enables bypass of oncogenic kras addiction in pancreatic cancer. Cell 2014;158:185–97.
25. Schroeder MC, Halder G. Regulation of the Hippo pathway by cell architecture and mechanical signals. Semin Cell Dev Biol 2012;23:803–11.
26. Zhao B, Li L, Wang L, Wang CY, Yu J, Guan KL. Cell detachment activates the Hippo pathway via cytoskeleton reorganization to induce anoikis. Genes Dev 2012;26:54–68.
27. Kaplan KB, Swedlow JR, Morgan DO, Varmus HE. c-Src enhances the spreading of src-/- fibroblasts on fibronectin by a kinase-independent mechanism. Genes Dev 1995;9:1505–17.
28. Cooper JA, Gould KL, Cartwright CA, Hunter T. Tyr527 is phosphorylated in pp60c-src: implications for regulation. Science 1986;231:1431–4.
29. Lippa B, Pan G, Corbett M, Li C, Kauffman GS, Pandit J, et al. Synthesis and structure based optimization of novel Akt inhibitors. Bioorg Med Chem Lett 2008;18:3359–63.
30. Li P, Silvis MR, Honaker Y, Lien WH, Arron ST, Vasioukhin V. alphaE-catenin inhibits a Src-YAP1 oncogenic module that couples tyrosine kinases and the effector of Hippo signaling pathway. Genes Dev 2016;30:798–811.
31. Kim NG, Gumbiner BM. Adhesion to fibronectin regulates Hippo signaling via the FAK-Src-PI3K pathway. J Cell Biol 2015;210:503–15.
32. Brown MT, Cooper JA. Regulation, substrates and functions of src. Biochim Biophys Acta 1996;1287:121–49.
33. Zhao B, Kim J, Ye X, Lai ZC, Guan KL. Both TEAD-binding and WW domains are required for the growth stimulation and oncogenic transformation activity of yes-associated protein. Cancer Res 2009;69:1089–98.
34. Dehm SM, Bonham K. SRC gene expression in human cancer: the role of transcriptional activation. Biochem Cell Biol 2004;82:263–74.
35. Codelia VA, Sun G, Irvine KD. Regulation of YAP by mechanical strain through Jnk and Hippo signaling. Curr Biol 2014;24:2012–7.

Published OnlineFirst July 28, 2017; DOI: 10.1158/0008-5472.CAN-17-0391

36. Dupont S, Morsut L, Aragona M, Enzo E, Giulitti S, Cordenonsi M, et al. Role of YAP/TAZ in mechanotransduction. Nature 2011;474:179–83.
37. Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, et al. Tensional homeostasis and the malignant phenotype. Cancer Cell 2005; 8:241–54.
38. Yu FX, Zhao B, Panupinthu N, Jewell JL, Lian I, Wang LH, et al. Regulation of the Hippo-YAP pathway by G-protein-coupled receptor signaling. Cell 2012;150:780–91.
39. Deng H, Wang W, Yu J, Zheng Y, Qing Y, Pan D. Spectrin regulates Hippo signaling by modulating cortical actomyosin activity. eLife 2015;4: e06567.
40. Fletcher GC, Elbediwy A, Khanal I, Ribeiro PS, Tapon N, Thompson BJ. The Spectrin cytoskeleton regulates the Hippo signalling pathway. EMBO J 2015;34:940–54.
41. Kono K, Tamashiro DA, Alarcon VB. Inhibition of RHO-ROCK signaling enhances ICM and suppresses TE characteristics through activation of Hippo signaling in the mouse blastocyst. Dev Biol 2014;394:142–55.
42. Yin F, Yu J, Zheng Y, Chen Q, Zhang N, Pan D. Spatial organization of Hippo signaling at the plasma membrane mediated by the tumor suppressor Merlin/NF2. Cell 2013;154:1342–55.
43. Rauskolb C, Sun S, Sun G, Pan Y, Irvine KD. Cytoskeletal tension inhibits Hippo signaling through an Ajuba-Warts complex. Cell 2014;158:143–56.
44. Sudol M. Yes-associated protein (YAP65) is a proline-rich phosphoprotein that binds to the SH3 domain of the Yes proto-oncogene product. Oncogene 1994;9:2145–52.
45. Taniguchi K, Wu LW, Grivennikov SI, de Jong PR, Lian I, Yu FX, et al. A gp130-Src-YAP module links inflammation to epithelial regeneration. Nature 2015;519:57–62.
46. Zaidi SK, Sullivan AJ, Medina R, Ito Y, van Wijnen AJ, Stein JL, et al. Tyrosine phosphorylation controls Runx2-mediated subnuclear targeting of YAP to repress transcription. EMBO J 2004;23:790–9.
47. Zhang S, Yu D. Targeting Src family kinases in anti-cancer therapies: turning promise into triumph. Trends Pharmacol Sci 2012;33:122–8.
48. Zhao Y, Yang X. The Hippo pathway in chemotherapeutic drug resistance. Int J Cancer 2015;137:2767–73.
49. Rosenbluh J, Nijhawan D, Cox AG, Li X, Neal JT, Schafer EJ, et al. β-Catenin-driven cancers require a YAP1 transcriptional complex for survival and tumorigenesis. Cell 2013;153:267–70.
50. Moroishi T, Hansen CG, Guan KL. The emerging roles of YAP and TAZ in cancer. Nat Rev Cancer 2015;15:73–9.

Published OnlineFirst July 28, 2017; DOI: 10.1158/0008-5472.CAN-17-0391

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Src Inhibits the Hippo Tumor Suppressor Pathway through Tyrosine Phosphorylation of Lats1

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