Negative Regulation of YAP by LATS1 Underscores Evolutionary Conservation of the *Drosophila Hippo* Pathway

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Abstract

The *Hippo* pathway defines a novel signaling cascade regulating cell proliferation and survival in *Drosophila*, which involves the negative regulation of the transcriptional coactivator *Yorkie* by the kinases *Hippo* and *Warts*. We have recently shown that the human ortholog of *Yorkie, YAP*, maps to a minimal amplification locus in mouse and human cancers, and that it mediates dramatic transforming activity in MCF10A primary mammary epithelial cells. Here, we show that LATS proteins (mammalian orthologs of *Warts*) interact directly with YAP in mammalian cells and that ectopic expression of LATS1, but not LATS2, effectively suppresses the YAP phenotypes. Furthermore, shRNA-mediated knockdown of LATS1 phenocopies YAP overexpression. Because this effect can be suppressed by simultaneous YAP knockdown, it suggests that YAP is the primary target of LATS1 in mammalian cells. Expression profiling of genes induced by ectopic expression of YAP or by knockdown of LATS1 reveals a subset of potential *Hippo* pathway targets implicated in epithelial-to-mesenchymal transition, suggesting that this is a key feature of YAP signaling in mammalian cells. [Cancer Res 2008;68(8):2789–94]

Introduction

Functional screens in *Drosophila* have identified a novel signaling pathway that regulates organ size by modulating cell growth, proliferation, and apoptosis (1, 2). Key components of this pathway are two serine/threonine kinases, *Hippo* and *Warts*, and the transcriptional coactivator *Yorkie*. Loss of function of *Hippo* or *Warts* results in increased proliferation and resistance to cell death (3–7). Overexpression of *Yorkie* phenocopies this effect, consistent with the negative regulation of *Yorkie* by *Warts* and *Hippo*. *Yorkie* activation is associated with increased expression of *cyclin E* and *DIAP*, potentially contributing to both proliferative and antiapoptotic effects (8). Two mammalian orthologs of *Drosophila Warts* have been identified: *LATS1* and *LATS2* (9–11). *Lats1*-deficient mice develop soft tissue sarcomas and ovarian stromal cell tumors (12). The *Lats2* knockout is embryonic lethal, but embryonic fibroblasts (mouse embryo fibroblast) show increased proliferative potential (13). Although the mammalian *Yorkie* ortholog, YAP, was originally identified as a binding partner for the Src family member YES (14), numerous additional interacting partners were subsequently described, including transcription factors polyomavirus encoding binding protein 2, the p53 family member p73, and TEA domain/transcription enhancer factor family members (15–17). YAP functions as a coactivator of these transcription factors in reporter assays.

Using array comparative genomic hybridization (CGH) in a mouse tumor model, we identified *YAP* as the "driver" gene in a small focal genomic amplification (18), which is syntenic to a larger multigene amplification present in human cancers of the pancreas, head and neck, ovary, cervix, and oral squamous cell carcinomas. We showed the transforming potential of YAP via proliferative and antiapoptotic activities in mammary epithelial cells (18), and similar oncogenic properties were shown in a mouse hepatocellular carcinoma model (19). Therefore, YAP represents a novel mammalian oncogene potentially regulated by an evolutionarily conserved kinase cascade. To test the conservation of the *Hippo* pathway in mammals, we analyzed the potential regulation of YAP by LATS proteins.

Materials and Methods

Cell culture. MCF10A cells were cultured as described (20). MDA-MB-231 cells were maintained in MEM supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 50 U/mL penicillin/streptomycin and incubated at 5% CO_2 at 37°C.

RNAi. siRNA duplexes targeting human *YAP* were purchased from Invitrogen. Sense strand sequence with dTdT overhang is listed in the 5′ to 3′ direction as follows: siYAP-1, GACAUCUUCUGGUCAGAGAdTdT; and siYAP-2, UCUCUGACCAGAAGAUGUCdTdT. Control siRNA is a scrambled sequence with no homology in the human genome (Qiagen) is listed as follows: Scrambled, UUCUCCGAACGUGUCACGUdTdT. siRNA duplexes were transfected using Lipofectamine2000 (Invitrogen), according to the manufacturer's instructions. Briefly, cells were plated and transfected the following day with siRNA duplexes at a final concentration of 40 nmol/L for 24 h without change of culture medium. The transfection was repeated on the second day under the same conditions. On the third day, cells were either harvested for RNA analysis or used for migration assays.

The shRNA hairpins against human *LATS1* and *YAP* were obtained from The RNAi Consortium (Broad Institute). Forward oligo sequence is listed in the 5′ to 3′ direction as follows: shLATS1-A, CCGGGTCTGCTTCATACATTCCTAACTCGAGTTAGGAATGTATGAAGCAGACTTTTT; shLATS1-B, CCGGGAGAAATTAAGCCATCGTGTTCTCGAGA ACACGATGGCTTAATTTCTCTTTTT; shYAP-3, CCGGCCCAGTTAAATGTTC ACCAATCTCGAGATTGGTGAACATTTAACTGGGTTTTTG; and shYAP-4, CCGGG CCACCAAGCTAGATAAAGAACTCGAGTTCTTTATCTAGCTTGGTGGCTTTTTG. Control shRNA was designed to target green fluorescent protein (GFP), a gene not expressed endogenously.

Lentivirus packaging, MCF10A cell transduction, and drug selection were performed following standard protocols.

Plasmid construction. The human YAP expression clone was described previously (18). The human LATS1 and LATS2 open reading frames were cloned into pBABE (hygromycin) vector as BglII/XhoI and BamHI/XhoI fragments, respectively. LATS1 was tagged with HA-tag on the NH_2 terminus, and LATS2 was tagged with myc-tag on the COOH terminus.

Antibodies. Phospho-AKT (Ser^{473}), phospho-AKT, phospho-YAP, and phospho-Myc antibodies were purchased from Cell Signaling Technology; YAP antibody from Santa Cruz biotechnology; β-actin antibody from

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

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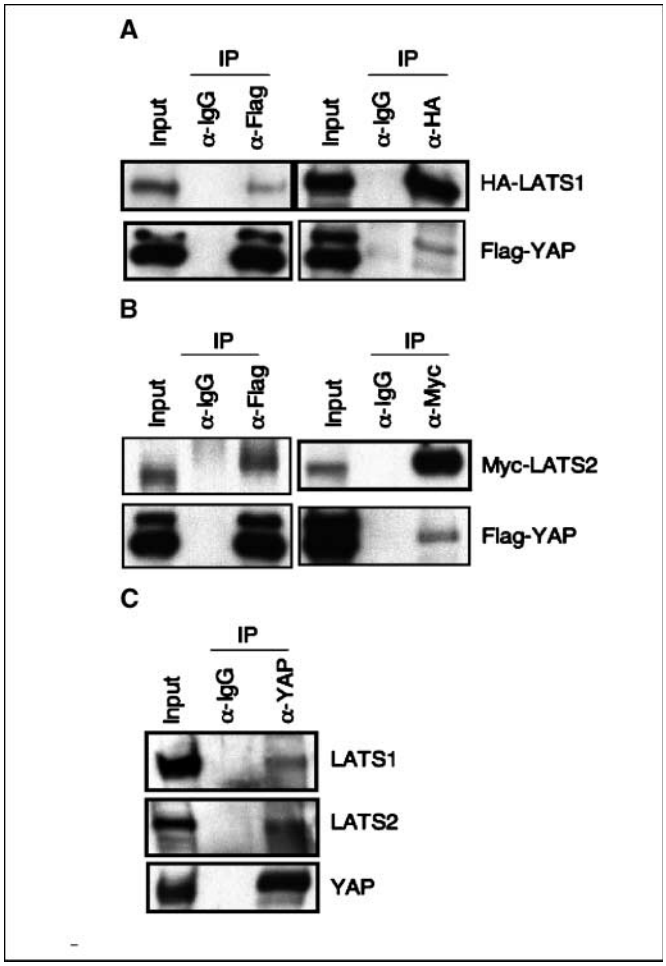

Figure 1. YAP interacts with both LATS1 and LATS2 *in vivo*. *A,* interaction of YAP and LATS1. Coimmunoprecipitation analysis of tagged YAP and LATS1 constructs after cotransfection into 293 cells. The coimmunoprecipitated proteins are compared with mock immunoprecipitates (*IgG*) and with the input lysate (10% of immunoprecipitated lysate). The coimmunoprecipitation/Western blot analyses were performed in both directions. *Left,* identification of LATS1 in the YAP precipitate; *right,* presence of YAP in the LATS1 immunoprecipitate (*IP*). *B,* interaction of YAP and LATS2. Coimmunoprecipitation of tagged transfected YAP and LATS2 constructs. *C,* coimmunoprecipitation of endogenous LATS1 and LATS2 with endogenous YAP. The immunoprecipitation/Western analysis was performed in untransfected HeLa cells.

Abcam; fibronectin and Flag (M2) antibodies from Sigma; E-cadherin, N-cadherin, and Vimentin antibodies from BD Biosciences; LATS1 and LATS2 antibodies from Bethyl, Inc.; and HA antibody from Roche Applied Science.

Cell migration and soft agar assays. Transwell cell migration assay and soft agar assay were performed as previously described (18).

Immunoprecipitation and Western blot. HEK293 cells were plated at 2×10^6 per 10-cm dish the day before transfection. Transient transfection was performed with 6 μg of total plasmid DNA/dish using FuGene6 transfection reagent (Roche). Expression of transfected genes was analyzed 48 h posttransfection. Cells were washed with PBS and collected with immunoprecipitation buffer [20 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 20% glycerol, 0.5% NP40, and 1× protease inhibitor cocktail (Complete EDTA-free; Roche)]. Cell lysates were cleared by centrifugation at 14,000 rpm for 20 min at 4°C. Thirty microliters of M2 anti–Flag-beads (Sigma), 5 μg anti-HA antibody (Roche), or 30 μL anti-Myc beads (Sigma) were added to the cleared lysates and incubated for 3 h at 4°C. Thirty microliters of protein G agarose bead suspension (Roche) were added to the anti-HA IP for 2 h at 4°C. Beads were washed with the immunoprecipitation buffer five times at 4°C before bound proteins were eluted with 2× SDS sample buffer and loaded onto 4% to 15% SDS-PAGE gel (ReadyGel; Bio-Rad). For immunoblotting analysis, proteins were transferred onto Immobilon polyvinylidene difluoride (Millipore), detected by various antibodies, and visualized with Western Lightning Plus chemiluminescence kit (Perkin-Elmer).

RNA preparation and quantitative real-time PCR detection. RNA was extracted using the RNeasy Mini kit (Qiagen). cDNA synthesis was performed using First-Strand cDNA Synthesis kit (GE Healthcare), and

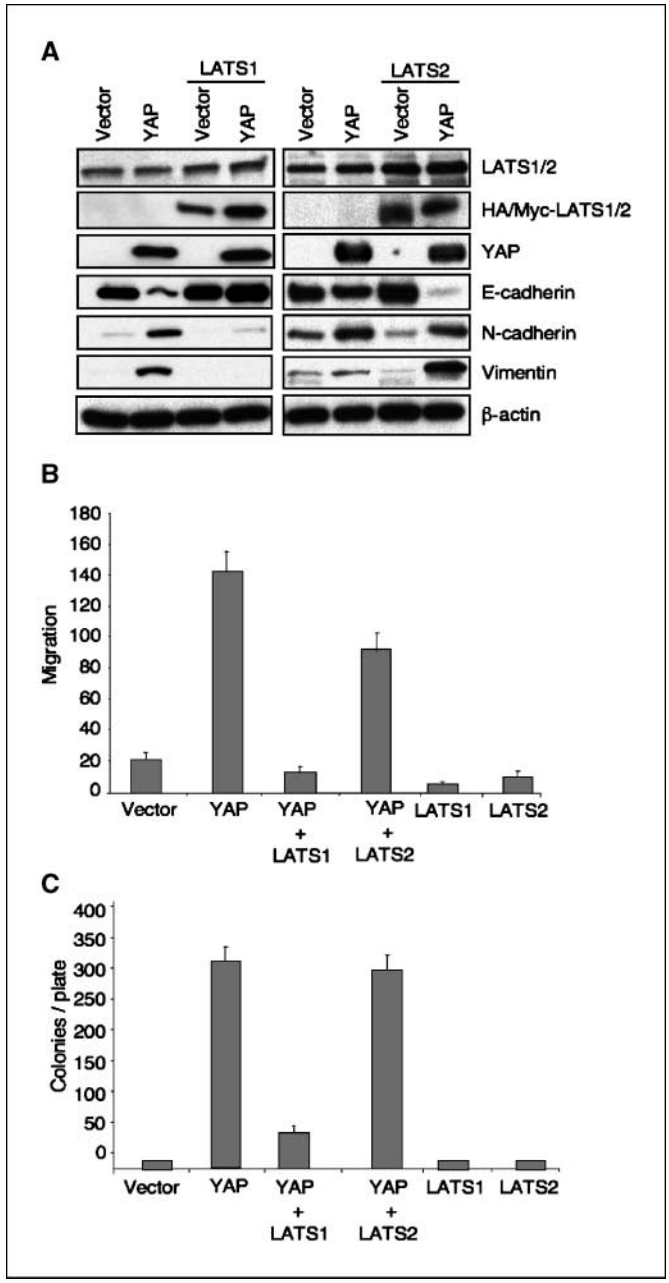

Figure 2. LATS1, but not LATS2, suppresses multiple YAP phenotypes. *A,* overexpression of LATS1, but not LATS2, suppresses YAP-induced EMT. Markers for epithelial (E-cadherin) and mesenchymal (N-cadherin and vimentin) states were used. Despite comparable overexpression levels of LATS family members, LATS2 had negligible effect on YAP-induced EMT. β-Actin was used as a loading control. *B,* overexpression of LATS1, but not LATS2, suppresses YAP-induced cell migration. *Columns,* mean number of migrated cells within four fields at ×20 magnification; *bars,* SD. *C,* overexpression of LATS1, but not LATS2, suppresses YAP-induced anchorage-independent growth in soft agar. *Columns,* mean number of colonies after 21 d in culture, calculated from 6 wells seeded with 50,000 cells; *bars,* SD.

quantitative real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems).

Sequence of the qPCR primer pairs (all listed in the 5′–3′ direction) were as follows:

GAPDH-F: GGTGAAGGTCGGAGTCAACGG;
GAPDH-R: GAGGTCAATGAAGGGGTCATTG;
YAP-F: CCTTCTTCAAGCCGCCGGAG;
YAP-R: CAGTGTCCCAGGAGAAACAGC;
LATS1-F: GAAACAAATTCTTCTCGGAGTACTTC;
LATS1-R: CTTCTGTTGTTAGTTTTCTGAAGAGC;
Fibronectin-F: GGCCAGACTCCAATCCAGAG;
Fibronectin-R: GGTAGATCTTGTAGTCAGTGC;
COL8A1-F: CAGAAACCAGCCCCAGAGGTGTCAC;
COL8A1-R: GAAATGGTAAGCAGCACTCCCAGCAG;
CTGF-F: GCAGAGCCGCCTGTGCATGG;
CTGF-R: GGTATGTCTTCATGCTGG;
CYR61-F: CACACCAAGGGGCTGGAATG;
CYR61-R: CCCGTTTTGGTAGATTCTGG;
E-cadherin–F: GAAGAGAGACTGGGTTATTCCTCCC;
E-cadherin–R: CAGTGATGCTGTAGAAAACCTTGCC;
N-cadherin–F: GGACAGCCTCTTCTCAATG;
N-cadherin–R: CTGCAGGCTCACTGCTCTC.

All measurements were performed in triplicate and standardized to the levels of *GAPDH*.

Microarray. RNA from ~60% confluent monolayers of MCF10A cells was harvested as described above. Vector- and YAP-expressing cells were compared in duplicate on Affymetrix Human Genome U133 Plus 2.0 arrays. Primary data are shown in Supplementary Table S1 and has been deposited in the National Center for Biotechnology Information Gene Expression Omnibus repository (GSE10196).

Results and Discussion

YAP interacts with LATS protein *in vivo*. We first analyzed the ability of LATS proteins to bind to YAP in cells transfected with tagged expression constructs. Flag-tagged YAP was coexpressed in 293 cells with either HA-LATS1 or Myc-LATS2. Coimmunoprecipitation of YAP and both LATS proteins is evident by immunoprecipitation and immunoblotting analysis using the antiepitope antibodies in either order (Fig. 1*A* and *B*). The association of endogenous YAP with LATS1 and LATS2 was confirmed in HeLa cells using antibodies to the endogenous proteins (Fig. 1*C*).

LATS1, but not LATS2, represses YAP phenotype. To test for functional interactions between the LATS proteins and YAP, we

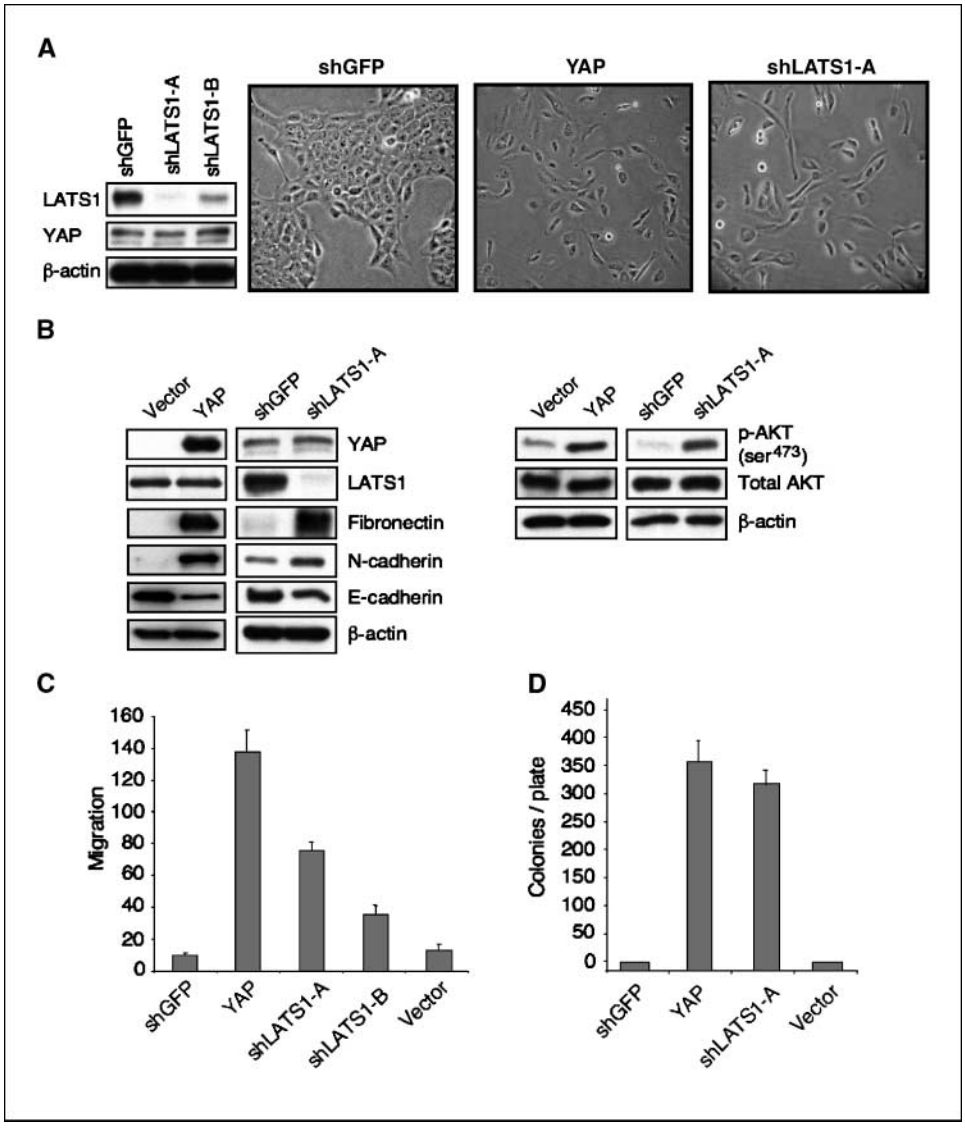

Figure 3. LATS1 knockdown phenocopies the effect of YAP overexpression. *A,* efficient knockdown of LATS1 using two independent lentiviral constructs (shLATS1-A and shLATS1-B). Although both shLATS1 constructs reduce LATS1 protein level and induce a change in the morphologic appearance of MCF10A cells, shLAST1-A is significantly better. shRNA hairpin–targeting GFP is used as a control. *B,* knockdown of LATS1 results in the induction of EMT and activation of the AKT pathway. Immunoblotting analysis reveals a decrease in E-cadherin (epithelial marker) and concomitant increase in N-cadherin and fibronectin (mesenchymal markers). AKT pathway was activated, as determined by phosphorylation status of AKT(Ser^{473}), upon reduction of LATS1 in MCF10A cells cultured in the absence of epidermal growth factor and low serum. β-Actin was used as a loading control. *C,* knockdown of LATS1 results in increased cell migration. *D,* knockdown of LATS1 results in the acquisition of anchorage-independent growth capacity by MCF10A cells.

ectopically expressed these by retroviral transduction into the immortalized, but nontransformed, human MCF10A breast epithelial cells. Drug-selected pools of infected cells were used in these analyses to avoid clonal selection effects. Immunoblotting analysis confirmed similar overexpression levels of LATS1 and LATS2 compared with their respective endogenous levels. Consistent with our previous observations (18), overexpression of YAP induced epithelial-to-mesenchymal transition (EMT), increased cell migration, and conferred anchorage-independent growth to MCF10A cells. Remarkably, coexpression of LATS1 effectively suppressed YAP-mediated induction of EMT, as evidenced by the diminished induction of mesenchymal markers, N-cadherin and vimentin, and by restoration of the epithelial marker E-cadherin (Fig. 2*A*). Comparable expression of LATS2, however, had no effect on EMT marker expression. Similarly, LATS1 abrogated the increased cell migration associated with YAP overexpression, whereas the effect of LATS2 was only modest (Fig. 2*B*). YAP-induced anchorage-independent colony formation was also profoundly suppressed by coexpression of LATS1 but not LATS2 (Fig. 2*C*). Taken together, these observations suggest that LATS1, but not LATS2, is a major regulator of the YAP phenotype.

Down-regulation of LATS1 phenocopies YAP phenotype. To determine whether LATS1 mediates a physiologic down-regulation of YAP activity, we knocked down the expression of LATS1 using a lentiviral shRNA construct. Effective knockdown of LATS1 was shown at the protein level, with no associated effect on YAP protein level (Fig. 3*A*). Although neither YAP overexpression nor LATS1 knockdown had an effect on the proliferation of MCF10A cells in monolayer cultures (data not shown), both resulted in a striking alteration in cellular morphology manifesting as a spindle-shaped mesenchymal appearance and increased cell scattering (Fig. 3*A*). Consistent with the EMT phenotype, expression of the mesenchymal markers N-cadherin and fibronectin was up-regulated by YAP overexpression or by LATS1 knockdown, whereas the epithelial marker, E-cadherin, was down-regulated by both of these manipulations (Fig. 3*B*). Results from several independent assays suggested that the LATS1 knockdown phenocopies YAP overexpression. First, reduction of LATS1 levels resulted in increased AKT phosphorylation in serum-starved MCF10A cells, similar to the effect of YAP overexpression (Fig. 3*B*). Second, LATS1 knockdown resulted in increased cell migration of MCF10A cells (Fig. 3*C*) and conferred anchorage-independent growth ability to MCF10A cells (Fig. 3*D*). Of note, however, the size of soft agar colonies associated with LATS1 knockdown was smaller than that associated with YAP overexpression. Collectively, these results strongly argue that LATS1 knockdown phenocopies YAP overexpression and suggest that LATS1 may be a critical endogenous negative regulator of YAP in mammalian cells.

Knockdown of YAP abrogates the effect of LATS1 knockdown. The striking effects of knocking down LATS1 may be due to loss of YAP regulation but could also be attributable to other targets of this kinase. To determine whether the LATS1 knockdown phenotype can be directly attributed to the relief of YAP inhibition, we tested the effect of knocking down YAP in cells with reduced LATS1 expression. The effective suppression of YAP protein was shown by immunoblotting (Fig. 4*A*). Remarkably, the phenotypes of LATS1 knockdown could be suppressed by concomitant YAP knockdown. siRNA targeting *YAP*, but not a control scrambled siRNA duplex, effectively suppressed the AKT activation associated with LATS1 knockdown (Fig. 4*B*). Similarly, increased migration of MCF10A associated with LATS1 knockdown was abrogated by the siRNA-mediated reduction of YAP levels (Fig. 4*C*). Taken together, these results suggest that YAP is the primary target of LATS1 kinase.

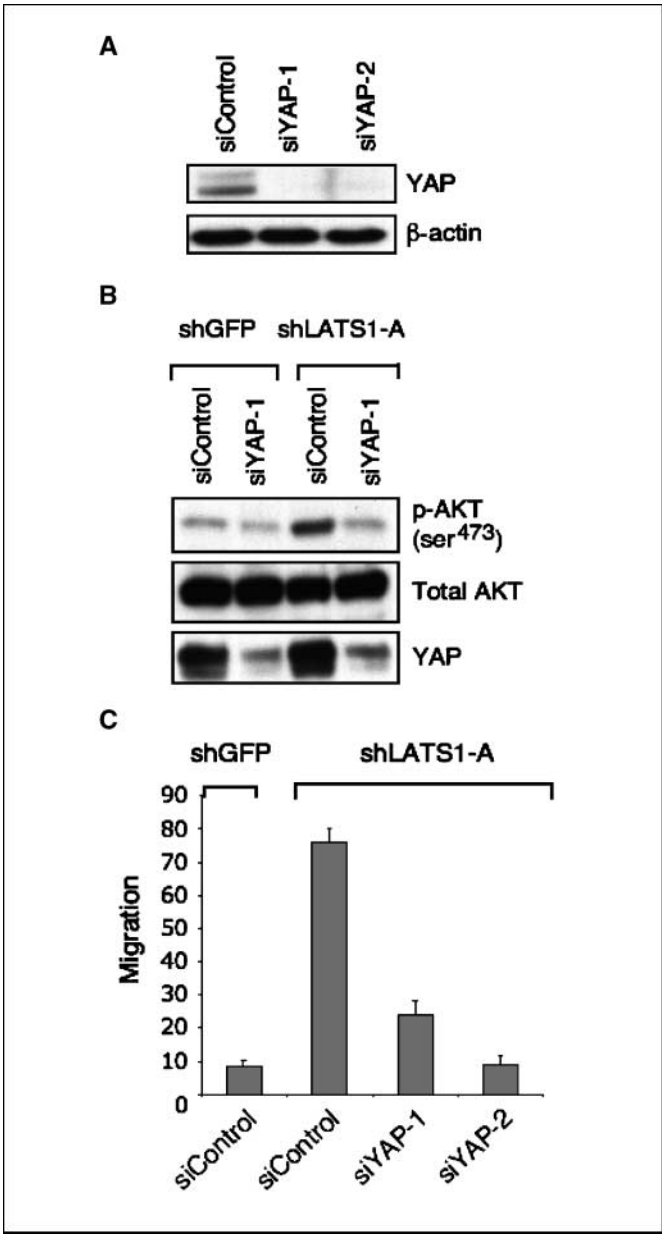

Figure 4. YAP is the primary target of LATS1 in MCF10A cells. *A,* efficient knockdown of YAP using two independent siRNA duplexes (siYAP-1 and siYAP-2) in MCF10A cells. siControl refers to a nonspecific siRNA duplex. β-Actin was used as a loading control. *B,* activation of AKT pathway due to knockdown of LATS1 is suppressed upon the reduction of YAP levels. Total AKT was used as a loading control. *C,* increased cell migration due to knockdown of LATS1 is suppressed upon the reduction of YAP levels.

Hippo pathway targets and EMT. The *Hippo* pathway is thought to converge on YAP, which in *Drosophila* regulates expression of *cyclin E, DIAP*, and the microRNA *Bantam* (8, 21, 22). However, analysis of MCF10A cells overexpressing YAP did not reveal altered expression of mammalian orthologs of these *Drosophila* YAP targets. To search for potential downstream targets of YAP, we therefore screened for genes whose expression was independently regulated by overexpression of YAP as well as by LATS1 knockdown.

We reasoned that this dual requirement would likely exclude secondary effects and enhance specificity of potential targets identified in this pathway. An initial screen consisted of comparing expression profiles of MCF10A cells overexpressing YAP and comparing them to vector control–transfected cells (Supplementary Table S1). Candidate genes were validated by real-time reverse transcription-PCR analysis in cells overexpressing YAP and in cells with LATS1 knockdown (Fig. 5*A*). The specificity of this approach was supported by the identification of coordinate regulation of known EMT markers, such as fibronectin, N-cadherin, and E-cadherin. In addition to these EMT markers, we identified several novel genes likely to be regulated by this pathway: Collagen VIII (*COL8A1*), connective tissue growth factor (*CTGF*), and cysteine-rich protein 61 (*CYR61*). It is interesting to note that these genes are associated with the extracellular matrix and have been previously described to be involved in modulating cell differentiation, adhesion, migration, and apoptosis (23, 24).

To confirm these candidate YAP target genes in another breast cancer cell line, we used MDA-MB-231, which expresses high levels of endogenous YAP (Fig. 5*B*). Endogenous YAP expression was efficiently knocked down using two independent lentiviral shRNA constructs. YAP knockdown significantly reduced the cell proliferation rate and reduced the migratory capacity of these cells (data not shown). Expression of the endogenous *COL8A1, CYR61*, and *CTGF* genes was also reduced by knockdown of endogenous YAP in these cells (Fig. 5*B*), suggesting that they may represent physiologic mammalian targets of the *Hippo* pathway.

Concluding remarks. In this study, we have shown that the mammalian orthologs of *Warts*, LATS1, and LATS2 associate with the mammalian ortholog of *Yorkie*, YAP. Three lines of evidence suggest that LATS1 is the physiologic regulator of YAP and that YAP is its primary target. First, overexpression of LATS1 effectively suppressed multiple phenotypes associated with YAP overexpression, such as expression of EMT markers, cell migration, and anchorage-independent growth. Second, knockdown of LATS1 effectively phenocopied YAP overexpression as judged by these phenotypes. Third, the phenotypes associated with LATS1 knockdown could be suppressed by the simultaneous reduction of YAP levels. The functional properties of mammalian LATS1 and YAP suggest a high degree of evolutionary conservation between the *Drosophila* pathway and its mammalian counterpart.

To further understand the molecular mechanisms of *Hippo* pathway activation in mammalian cells, we identified a group of genes regulated both by YAP overexpression and by LATS1 knockdown in MCF10A cells. While this article was in preparation, a transgenic mouse model conditionally overexpressing YAP in the liver was reported; showing that the mammalian *Hippo* pathway is a potent regulator of organ size whose dysregulation leads to tumorigenesis (25, 26). Interestingly, the genes we identified as YAP targets show partial overlap with the YAP targets from the transgenic liver model including *CTGF* and *CYR61*. Transcriptional targets of the Hippo pathway in mammalian cells may thus include tissue-specific genes as well as a core set of downstream effectors regulating fundamental cellular phenotypes.

The induction of EMT by YAP overexpression is consistent with an emerging concept of EMT inducers as oncogenes. In fact, *YAP* amplification is observed in human cancers of the head and neck, pancreas, lung, ovary, cervix, and oral squamous-cell carcinomas (27–32). During embryonic development, EMT allows cells to traverse great distances to reach their final destination, and presumably activate antiapoptotic responses to avoid anoikis, the characteristic cell death program induced by detachment of epithelial cells. The induction of EMT by YAP is not a result from its regulation of known transcriptional modulators of EMT, such as *SNAIL, SLUG, E2A*, or *TWIST* (data not shown), suggesting that this effect is a direct result of a YAP-dependent transcriptional

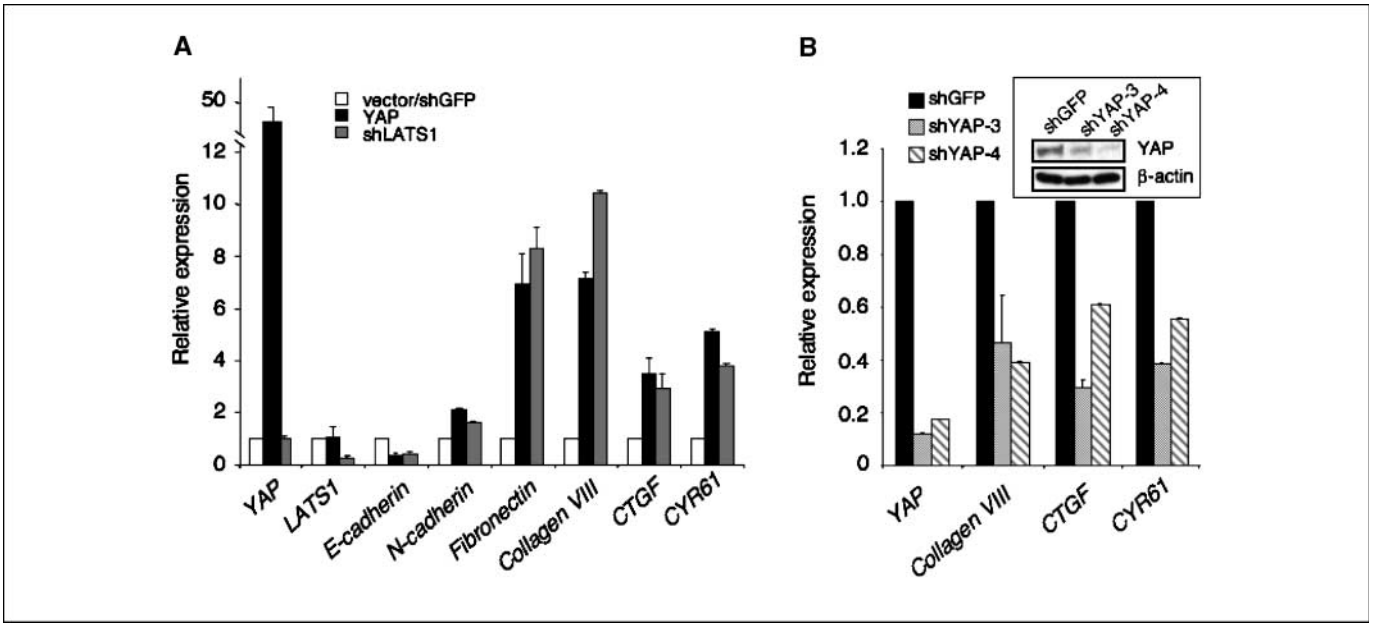

Figure 5. Candidate YAP target genes. *A,* qPCR analysis of candidate YAP target genes in MCF10A cells. Similar effects on target gene expression were observed upon YAP overexpression or LATS1 knockdown. *GAPDH* was used as an internal control. *Columns,* mean; *bars,* SD. *B,* qPCR analysis of candidate YAP target genes in MDA-MB-231 cells. *Insert,* efficient knockdown of endogenous YAP using two independent lentiviral shRNA constructs (shYAP-3 and shYAP-4). ShRNA-targeting GFP was used as a hairpin control. β-Actin was used as an immunoblotting loading control. Knockdown of YAP expression resulted in a significant decrease of target gene expression. *Columns,* mean; *bars,* SD.

program. The observation that LATS1 knockdown alone is sufficient for EMT suggests that the mammalian *Hippo* pathway is an endogenous EMT regulatory pathway.

LATS1/2 belong to the NDR (nuclear Dbf2-related) protein-kinase family that are essential components of pathways controlling key cellular processes, including morphologic changes, mitotic exit, cytokinesis, cell proliferation, and apoptosis (33). Although mutations in these genes seem to be uncommon in human cancer cell lines (data not shown), the promoters of *LATS1* and *LATS2* seem to be hypermethylated in human breast cancers that have larger tumor size, a higher likelihood of lymph node metastases, and estrogen receptor and progesterone receptor negativity (34). Upstream components of the Hippo pathway, including the human ortholog of *Salvador, SAV1* (5), the Neurofibromatosis type II gene, *NF2* (35), and *RASSF1* (36) are also disrupted in subsets of human cancers. Thus, although the full effect of the Hippo pathway on human malignancy awaits a full delineation of its components and comprehensive mutational analyses, its functional properties in model organisms and its high degree of evolutionary conservation suggest it is an important pathway directing EMT and cellular proliferation.

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