Cancer Research

# Both TEAD-Binding and WW Domains Are Required for the Growth Stimulation and Oncogenic Transformation Activity of Yes-Associated Protein

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*Cancer Res* 2009;69:1089-1098. Published OnlineFirst January 13, 2009.

Published OnlineFirst January 13, 2009; DOI:10.1158/0008-5472.CAN-08-2997

Research Article

# Both TEAD-Binding and WW Domains Are Required for the Growth Stimulation and Oncogenic Transformation Activity of Yes-Associated Protein

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## Abstract

**The Yes-associated protein (YAP) transcription coactivator is a candidate human oncogene and a key regulator of organ size. It is phosphorylated and inhibited by the Hippo tumor suppressor pathway. TEAD family transcription factors were recently shown to play a key role in mediating the biological functions of YAP. Here, we show that the WW domain of YAP has a critical role in inducing a subset of YAP target genes independent of or in cooperation with TEAD. Mutation of the WW domains diminishes the ability of YAP to stimulate cell proliferation and oncogenic transformation. Inhibition of YAP oncogenic-transforming activity depends on intact serine residues 127 and 381, two sites that could be phosphorylated by the Hippo pathway. Furthermore, genetic experiments in *Drosophila* support that WW domains of YAP and Yki, the fly YAP homologue, have an important role in stimulating tissue growth. Our data suggest a model in which YAP induces gene expression and exerts its biological functions by interacting with transcription factors through both the TEAD-binding and WW domains.** [Cancer Res 2009;69(3):1089–98]

## Introduction

Yes-associated protein (YAP) is a transcription coactivator and a candidate human oncogene regulated by the Hippo pathway, a novel tumor suppressor pathway first characterized by *Drosophila* genetic studies (1–10). The Hippo pathway limits organ size in *Drosophila* by inhibiting Yki, the YAP homologue (11). Biochemical studies showed that Yki is directly phosphorylated and inhibited by the Wts protein kinase, which is phosphorylated and activated by the Hippo protein kinase (12, 13). Components of the Hippo pathway are highly conserved in mammals. Recent studies from our group and others have shown that YAP is phosphorylated and inhibited by the Lats tumor suppressor kinase, which is the mammalian homologue of Wts (14–16). Lats phosphorylates YAP on serine residue 127 in the HXRXXS motif, which results in 14-3-3 binding and cytoplasmic retention of YAP, therefore leading to YAP inhibition (14). This mechanism of YAP regulation is implicated in cell contact inhibition and tissue growth control (14, 17).

YAP is a potent growth promoter. Overexpression of YAP increases organ size in *Drosophila* and saturation cell density in NIH-3T3 cell culture (14). However, *yap* was termed a candidate oncogene only after it was shown to be in human chromosome 11q22 amplicon that is evident in several human cancers (18–21). Besides the genomic amplification, YAP expression and nuclear localization were also shown to be elevated in multiple types of human cancers (12, 14, 20, 22). Several experiments further confirmed that YAP has oncogenic function: YAP overexpression in MCF10A cells induces epithelial-mesenchymal transition (EMT), which is often associated with cancer metastasis (21); YAP cooperates with *myc* oncogene to stimulate tumor growth in nude mice (20); and more interestingly, transgenic mice with liver-specific YAP overexpression show a dramatic increase in liver size and eventually develop tumors (12, 23). The above evidence strongly indicates the function of *yap* as an oncogene, although the mechanism by which YAP promotes oncogenesis is a question that remains to be answered.

YAP is a transcription coactivator, which itself has no DNA-binding activity. Recent studies from *Drosophila* and mammalian cells have shown that TEAD plays a critical role in mediating YAP-dependent gene induction and growth control (24–28). YAP and TEAD bind to a common set of promoters in MCF10A cells (27). Disruption of YAP-TEAD interaction or knockdown of TEAD attenuates the expression of many YAP target genes and blocks YAP-induced growth promotion and EMT (27). The *Drosophila* TEAD homologue, Scalloped (Sd), also interacts with Yki and is required for Yki to stimulate tissue growth (24–26). Collectively, TEAD is a key downstream transcription factor mediating YAP cellular function. However, in *Drosophila, yki* mutant cells have more severe growth defects than *sd* mutant cells (11, 29, 30), and overexpression of the Sd-binding–defective Yki-S97A elicits a reduced but still obvious overgrowth in *Drosophila* eyes and wings (27). Consistently, the TEAD-binding–defective YAP-S94A mutant can still induce expression of a fraction of YAP-regulated genes (27). These observations indicate that besides TEAD, additional transcription factors may be used by YAP/Yki to stimulate cell and tissue growth.

YAP has an $NH_2$-terminal TEAD-binding domain (TBD) and a COOH-terminal transactivation domain, with one or two WW domains (two splicing variants, YAP1 and YAP2, respectively) in between (31). The WW domain is a protein-protein interaction module with two signature tryptophan (W) residues spaced 20 to 22 amino acids apart (32). It binds to ligands containing proline-rich sequences. For example, the PPXY motif represents the largest class of WW domain ligands. Interestingly, PPXY motif is present in a wide range of transcription factors, among which ErbB4 intracellular domain (33), RUNX2 (34), and p73 (35, 36) have already been reported to bind to YAP WW domain. However, it is

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

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doi:10.1158/0008-5472.CAN-08-2997

Published OnlineFirst January 13, 2009; DOI:10.1158/0008-5472.CAN-08-2997



not clear if the WW domain, therefore any of the PPXY motif-containing transcription factors, mediates the gene induction and biological functions of YAP. The Lats kinase, which regulates YAP activity by direct phosphorylation, also contains one or two PPXY motifs (Lats2 has one and Lats1 has two PPXY). Therefore, the WW domain of YAP was also suggested to contribute to YAP inhibition by mediating interaction with Lats (15, 37).

In this report, we show that the WW domain of YAP is not essential for its inhibition by Lats. However, it is critical for induction of a subset of YAP target genes in cooperation with or independent of TEAD. Mutation of the WW domains diminishes the ability of YAP to promote cell proliferation, serum-independent growth, and oncogenic transformation. Interestingly, the WW domain is not essential for YAP to induce EMT in MCF10A cells, whereas TBD is required for both cell proliferation and EMT. The phosphorylation-defective YAP-5SA mutant is capable of transforming NIH-3T3 cells, and its oncogenic activity is inhibited by restoring either one of serine residues 127 or 381. Moreover, genetic experiments in *Drosophila* show a critical role of WW domains of YAP and Yki in stimulating tissue growth *in vivo*. Our study suggests that transcription factors interacting with the WW domains of YAP play an important role in mediating the oncogenic and growth promotion function of YAP.

## Materials and Methods

**Cell culture, transfection, and retroviral infection.** HEK293 cells, HEK293-T cells, and NIH-3T3 cells were cultured in DMEM (Invitrogen) containing 10% fetal bovine serum (FBS; Invitrogen) and 50 μg/mL penicillin/streptomycin. MCF10A cells were cultured in DMEM/F12 (Invitrogen) supplemented with 5% horse serum (Invitrogen), 20 ng/mL epidermal growth factor (EGF), 0.5 μg/mL hydrocortisone, 10 μg/mL insulin, 100 ng/mL cholera toxin, and 50 μg/mL penicillin/streptomycin. Transfection with Lipofectamine was performed according to the manufacturer's instructions.

To generate stable cells expressing wild-type (WT) or the indicated mutant YAP proteins, retrovirus infection was performed by transfecting 293 Phoenix retrovirus packaging cells with empty vector or pQCXIH-YAP constructs. Forty-eight hours after transfection, retroviral supernatant was supplemented with 5 μg/mL polybrene, filtered through a 0.45-μm filter, and used to infect MCF10A or NIH-3T3 cells. Thirty-six hours after infection, cells were selected with 200 μg/mL hygromycin (Roche) in culture medium.

**Three-dimensional culture of MCF10A cells.** The three-dimensional culture of MCF10A cells was done as described elsewhere (38). Briefly, growth factor–reduced Matrigel was layered onto eight-well glass chamber slide to make a reconstituted basement membrane. MCF10A cells were seeded on top of that at a concentration of 5,000 per well in assay medium containing 2% Matrigel and 5 ng/mL EGF. Cells were cultured in a 5% $CO_2$ humidified incubator at 37°C. The medium was replaced every 4 d.

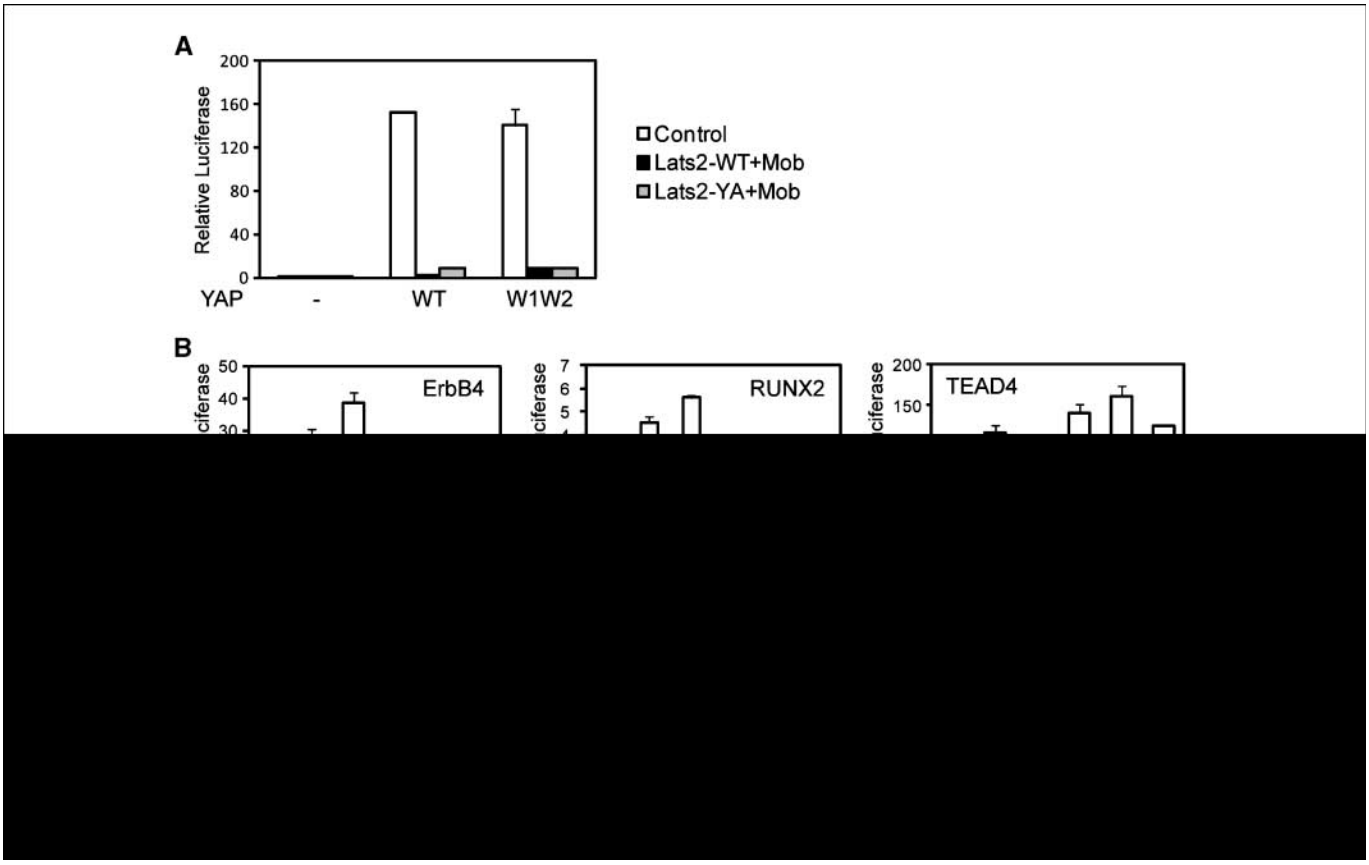


**Figure 1.** WW domains of YAP mediate gene induction but are not required for YAP inhibition by Lats. *A,* WW domains of YAP are not required for the inhibition by Lats2. Indicated plasmids were cotransfected with a 5× UAS-luciferase reporter, Gal4-TEAD4, and a CMV-β-gal construct into 293T cells. Luciferase activity was measured and normalized to β-galactosidase activity. W1W2 denotes mutation of the two WW domains in YAP; Lats2-YA denotes the Lats2 PPXY motif mutant. *B,* the TBD and WW domains mediate the activation of different transcription factors by YAP. YAP WT or mutants were cotransfected with the indicated transcription factors into 293T cells. ErbB4 intracellular domain and TEAD4 were Gal4 fused and were cotransfected with a 5× UAS-luciferase reporter. RUNX2 was cotransfected with the 6× OSE2-luciferase reporter. Luciferase activity was measured and normalized to activity of cotransfected β-galactosidase. *C,* both the TBD and WW domains are involved in YAP-induced gene expression. MCF10A cells stably expressing YAP WT or mutants were generated by retroviral infection. The expression of indicated genes was determined by quantitative reverse transcription-PCR and compared with vector control cells.

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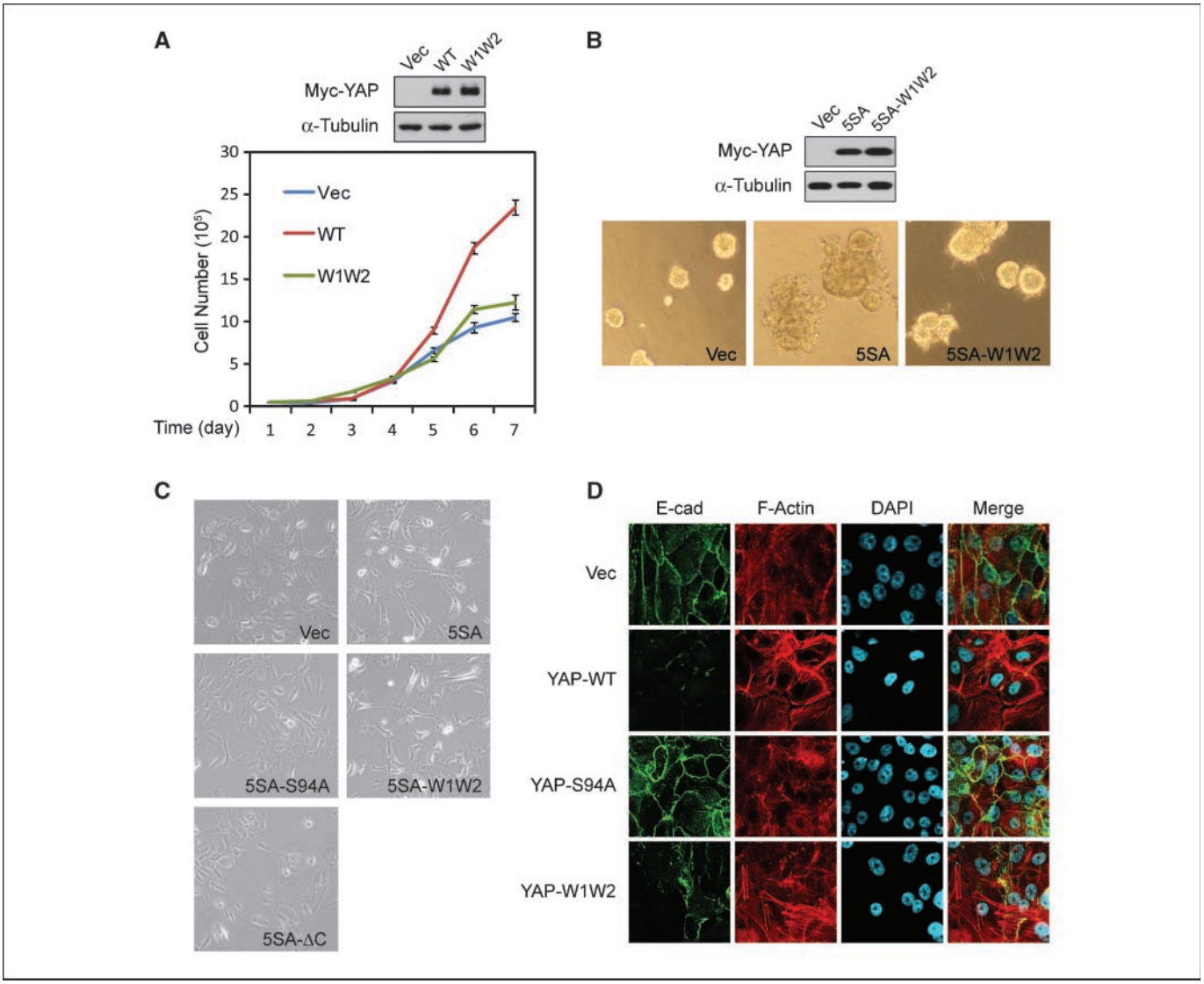


**Figure 2.** The WW domain is required for YAP-induced overgrowth but not EMT. *A,* YAP-W1W2 is defective in promoting cell growth. Growth curve of NIH-3T3 stable cells with expression of vector, YAP, and YAP-W1W2 was determined. *Top,* the expression of YAP WT or W1W2 mutant was shown by Western blot. *B,* WW domain mutant of YAP is comprised in inducing enlarged acini of MCF10A cells in three-dimensional culture. Indicated MCF10A stable cells were cultured in 3D on reconstituted basement membrane for 16 d before pictures were taken. *Top,* the ectopic expression of YAP was shown by Western blot. *C,* WW domains of YAP are not required for inducing an EMT-like morphology in MCF10A cells. The morphology of indicated MCF10A stable cells in tissue culture was recorded to show their difference. *D,* the TBD but not WW domain is required for reducing membrane E-cadherin and cortical actin. Indicated MCF10A stable cells were stained by anti-E-cadherin (*green*), rhodamine-phalloidin (*red*), and 4′,6-diamidino-2-phenylindole (*DAPI*; *blue*).

**Luciferase assay.** For the luciferase reporter assay, HEK293-T cells were seeded in 12-well plates. Luciferase reporter, cytomegalovirus (CMV)-β-gal, and indicated plasmids were cotransfected. Thirty-six hours after transfection, cells were lysed and luciferase activity was assayed using the enhanced luciferase assay kit obtained from BD Biosciences following the manufacturer's instructions. All luciferase activities were normalized to β-galactosidase activity.

**RNA isolation and real-time PCR.** Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen). cDNA was synthesized by reverse transcription using random hexamers and subjected to real-time PCR with gene-specific primers in the presence of SYBR Green (Applied Biosystems). Relative abundance of mRNA was calculated by normalization to hypoxanthine phosphoribosyltransferase 1 mRNA.

**Colony formation assay.** Colony formation assay was performed as briefly described below. NIH-3T3 fibroblasts were seeded on six-well plates at a density of $10^5$ cells per well and then transfected with YAP WT or mutants using Fugene6 (Roche) according to the manufacturer's instructions. After 2 d, cells were replated onto 10-cm dish and maintained in DMEM supplemented with 5% FBS for 2 to 3 wk until foci were evident. Cells were fixed with 10% acetic acid and 10% methanol, and then colonies were stained with 1% crystal violet and counted.

***In vitro* kinase assay.** For Lats2 kinase assays, HEK293 cells were cotransfected with HA-Lats2 and Flag-Mst2 to express active Lats protein. Forty-eight hours after transfection, cells were lysed with lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, 10 mmol/L pyrophosphate, 10 mmol/L glycerophosphate, 50 mmol/L NaF, 1.5 mmol/L $Na_3VO_4$, protease inhibitor cocktail (Roche), 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride] and immunoprecipitated with anti-HA antibodies. The immunoprecipitates were washed thrice with lysis buffer, once with buffer containing 40 mmol/L HEPES and 200 mmol/L NaCl, and once with kinase assay buffer (30 mmol/L HEPES, 50 mmol/L potassium acetate, 5 mmol/L $MgCl_2$). The immunoprecipitated Lats2 was subjected to a kinase assay in the presence of 500 μmol/L cold ATP, 10 μCi [$\gamma$-$^{32}$P]ATP, and 1 μg of bacterially expressed WT or mutant glutathione

Published OnlineFirst January 13, 2009; DOI:10.1158/0008-5472.CAN-08-2997



*S*-transferase (GST)-YAP proteins as substrate at 30°C for 30 min with a gentle shaking. The reaction was terminated by adding SDS sample buffer and subjected to SDS-PAGE and autoradiography.

***Drosophila* genetics.** For *in vivo* functional analysis of YAP/Yki, full-length cDNAs of *YAP* or *yki* were cloned into a transformation vector *pUAST* (39). Multiple transgenic fly lines were generated for each of the following DNA constructs: *pUAS-Flag-YAP*$^{S127A/W1W2}$ (15 lines) and *pUAS-yki*$^{W1W2}$*-V5* (31 lines). *pUAS-Flag-YAP*$^{S127A}$ and *pUAS-yki-V5* were previously reported (14). *C5-Gal4* and *GMR-Gal4* drive wing- and eye-specific expression of *UAS* transgenes, respectively. For adult wing size analysis, at least 30 wings of each genotype were used for analysis. For clonal overexpression analysis of Yki and YAP, corresponding UAS transgenic flies were crossed with *w, hsFLP; act>y+>Gal4; UAS-GFP/TM6B* and progenies were raised at 20°C. Four days later, the flies were heat treated at 31°C for 1 h and then left at 20°C for another 3 d. Late third instar larvae were dissected and wing imaginal discs were fixed in 8% paraformaldehyde-lysine-phosphate buffer for 45 min at 4°C. Green fluorescent protein (GFP) signal was observed by confocal microscopy. Immunofluorescent staining of mid-pupal eye discs was done with mouse anti-Discs large (Dlg; 1:300; Developmental Studies Hybridoma Bank) as primary antibody and Alexa Fluor 488 (1:300; Molecular Probes) as secondary antibody. Scanning electron microscopy was done to reveal adult retinal phenotypes.

## Results

**WW domains are not required for YAP inhibition by Lats.** It has been suggested that the WW domains of YAP may bind to the PPXY motifs of Lats, therefore playing a role in recruiting Lats to YAP (15, 37). To test this possibility, we examined the effect of Lats on YAP WW domain mutant in reporter assay. Our data show that with Mob cotransfection, Lats could potently inhibit both WT and WW domain mutant YAP (Fig. 1*A*), indicating that the WW domains of YAP are not required for its inhibition by Lats. Similar results were obtained without Mob cotransfection, although the inhibition on both YAP-WT and W1W2 is less potent (data not shown). Consistently, mutation of the PPXY motif in Lats2 did not abolish its ability to inhibit YAP (Fig. 1*A*). These results argue against a model in which the WW domain mediates the inhibition of YAP by Lats.

**Both the TBD and WW domains of YAP are involved in gene induction.** It is possible that WW domains of YAP mediate interactions with transcription factors, therefore regulating gene expression. Several transcription factors, such as ErbB4 and

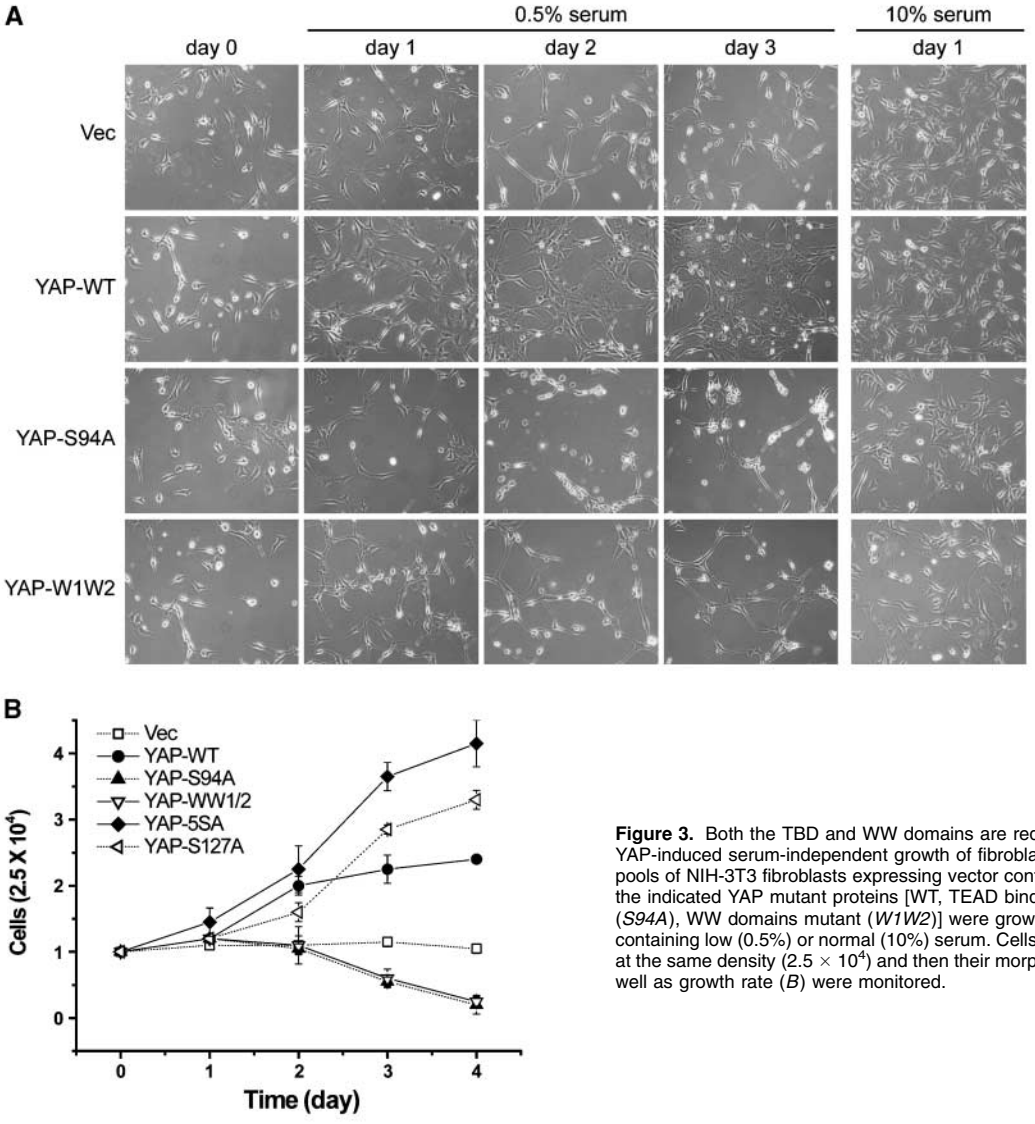


**Figure 3.** Both the TBD and WW domains are required for YAP-induced serum-independent growth of fibroblasts. Stable pools of NIH-3T3 fibroblasts expressing vector control (*Vec*) and the indicated YAP mutant proteins [WT, TEAD binding defective (*S94A*), WW domains mutant (*W1W2*)] were grown in medium containing low (0.5%) or normal (10%) serum. Cells were seeded at the same density ($2.5 \times 10^4$) and then their morphology (*A*) as well as growth rate (*B*) were monitored.

Published OnlineFirst January 13, 2009; DOI:10.1158/0008-5472.CAN-08-2997



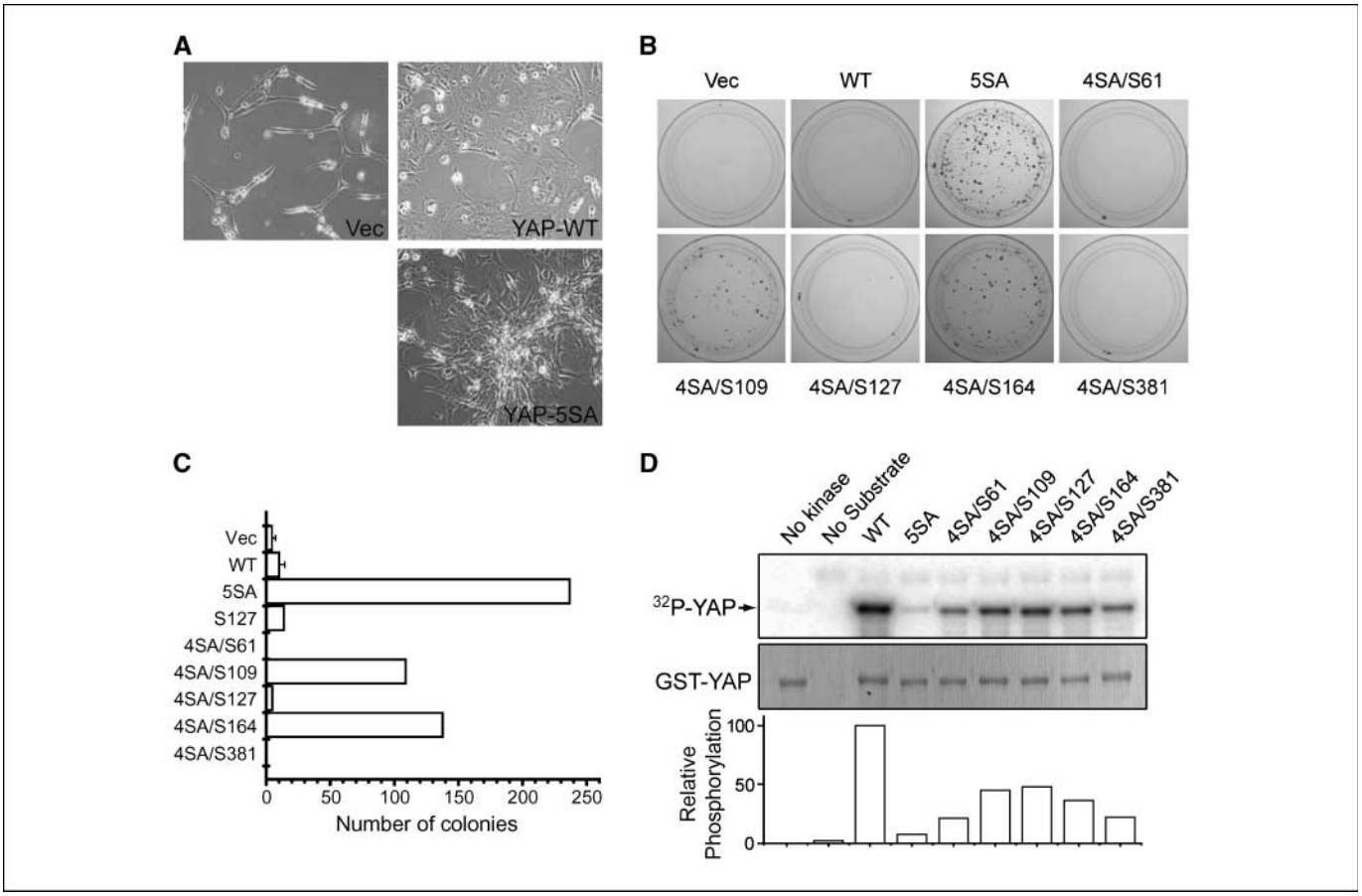


**Figure 4.** Phosphorylation of serine 127 or 381 is sufficient to inhibit transformation potential of YAP. *A,* YAP-5SA elicits a transformed morphology in NIH-3T3 cells. NIH-3T3 fibroblasts expressing vector (*Vec*), YAP-WT, or 5SA were seeded at the same density in medium containing 0.5% serum and their morphology after 4 d was shown. *B,* serine 127 and 381 were sufficient to confer inhibition of YAP-induced colony formation. Colony formation assays were performed using vector control or indicated YAP constructs. Colonies were visualized with crystal violet staining and pictured. The absence of colonies in the plate of YAP-4SA/S61 transfected cells is likely due to lack of YAP-4SA/S61 expression (data not shown). *C,* quantification of the colony number shown in *B*. Colony number in assay using YAP-S127A is also shown. *D,* all five HXRXXS motifs of YAP could be phosphorylated by Lats *in vitro*. WT YAP and various phosphorylation mutants were purified from bacteria as GST fusion proteins and were subjected to kinase assays in the presence of [$^{32}$P]ATP with immunoprecipitated Lats from HEK293 cells. Phosphorylation of YAP was detected by $^{32}$P incorporation (*top*) and GST-YAP input was shown by Coomassie blue staining (*middle*). *Bottom,* the relative $^{32}$P incorporation was quantified.

RUNX2, have been reported to be activated by YAP (33, 34). We examined the involvement of different domains of YAP in activation of these transcription factors. We previously identified serine 94 of YAP as an essential residue for its interaction with TEADs (27). As expected, S94A mutation of YAP completely abolished its ability to activate TEAD4 (Fig. 1*B*). Interestingly, YAP S94A mutant is capable of fully activating both ErbB4 and RUNX2, indicating that the TBD of YAP is not involved in its interaction with either ErbB4 or RUNX2. YAP has two WW domains, the first one of which has been implicated in interaction with ErbB4 and RUNX2 (33, 34). We found that mutation of the first (W1) or both (W1W2) WW domains in YAP abolished its ability to activate ErbB4 or RUNX2, whereas mutation of the second WW domain (W2) only modestly decreased this activity (Fig. 1*B*). However, mutation of the WW domains does not attenuate the activity of YAP on TEAD4. These data indicate that YAP uses two distinct domains, the TBD and WW, to activate different downstream target transcription factors.

As we previously reported, the TBD of YAP is required for induction of many YAP-inducible genes in MCF10A cells (27). Here, we compared gene expression profiles of MCF10A cells over-expressing YAP WT or WW domain mutant. Interestingly, a subset of YAP-inducible genes requires the intact WW domains in YAP (Supplementary Table S1). The expression of some of those genes was confirmed by real-time PCR as shown in Fig. 1*C*. Induction of ALPP largely depends on the WW domains but not the TBD. In contrast, induction of CTGF is absolutely dependent on TBD but not WW domains. Moreover, induction of ITGB2 and PIK3C2B requires both the TBD and WW domains (Fig. 1*C*). Therefore, it is clear that WW domains are essential for the expression of a subset of YAP-inducible genes, some of which also depend on the TBD.

**The WW domain is required for YAP-induced proliferation but not EMT.** YAP expression stimulates cell growth in both NIH-3T3 fibroblast and MCF10A (21, 27), a human mammary epithelial cell line. We tested the function of YAP WW domains in stimulating cell growth. Stable expression of WT YAP significantly increased NIH-3T3 cell growth compared with the vector control cells (Fig. 2*A*). However, expression of YAP-W1W2 mutant failed to do so. The effect of YAP expression on MCF10A cell growth was assayed in three-dimensional culture on reconstituted basement membrane. Expression of YAP-5SA, an active mutant with elimination of all five HXRXXS phosphorylation sites, strongly increased the acini size in three-dimensional culture (Fig. 2*B*). In

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contrast, mutation of WW domains significantly attenuated this activity of YAP-5SA.

Previously, it had been reported that YAP expression promotes EMT in MCF10A cells (27). We compared the cell morphology of MCF10A cells stably expressing YAP-5SA or YAP-5SA-W1W2. Surprisingly, cells expressing YAP-5SA-W1W2 display EMT-like morphologic changes similar to those induced by YAP-5SA (Fig. 2*C*). In contrast, mutation of S94 or deletion of the COOH-terminal activation domain abolished this activity of YAP-5SA. YAP-induced EMT in MCF10A cells was also shown by the loss of cell-cell junction localized E-cadherin and the switch from cortical actin to stress fibers (Fig. 2*D*). These alterations were induced by WT YAP as well as YAP-W1W2 but not YAP-S94A mutant (Fig. 2*D*). Our results suggest that the WW domain is not required for YAP to induce EMT but is important for YAP to promote proliferation in MCF10A cells.

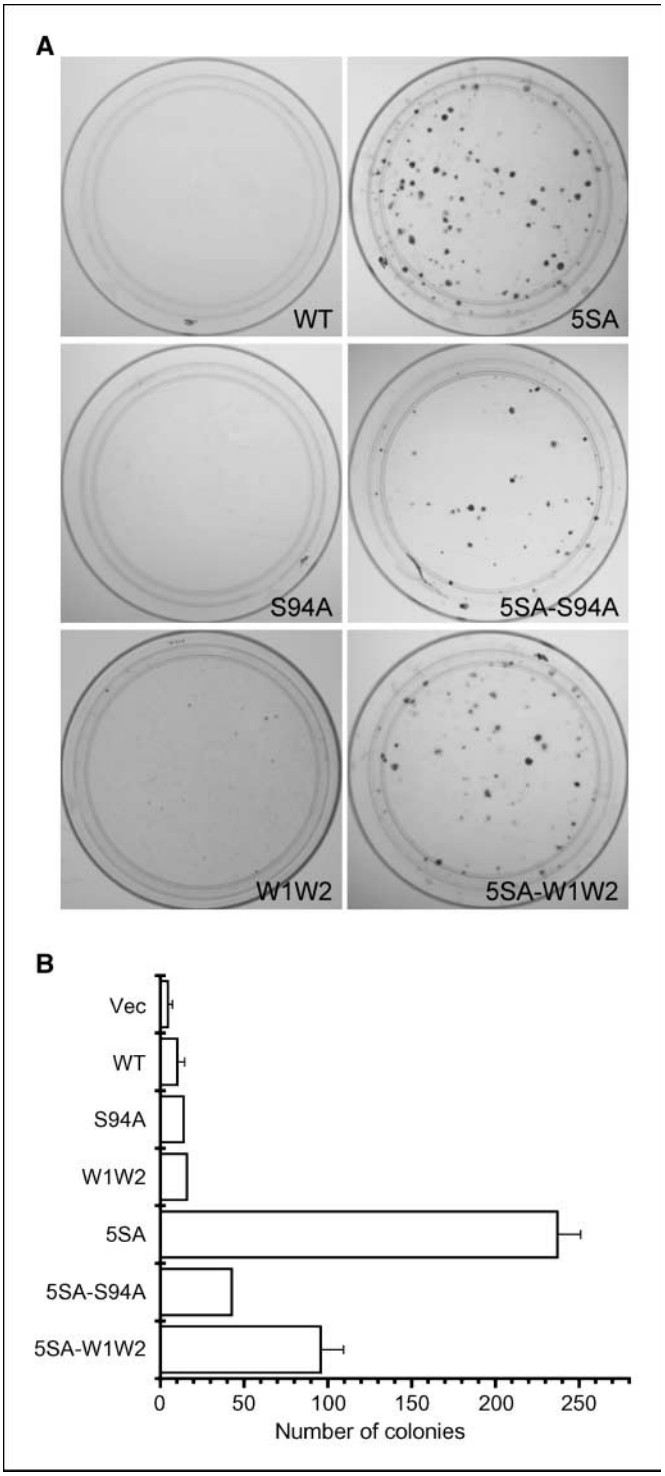


**Figure 5.** Both the TBD and WW domains are important for the oncogenic activity of YAP. Colony formation assay was performed with indicated plasmids. Colonies were stained with crystal violet and then pictured (*A*) and counted (*B*).

**Both the TBD and WW domains are required for cell growth in low serum medium.** YAP is a candidate oncogene capable of promoting tumor formation, which requires the cell to not only proliferate faster but also gain other characters, such as self-sufficiency of growth signals, a hallmark of cancer (40). We tested the ability of YAP WT or mutants to induce NIH-3T3 cell serum-independent growth. In medium containing 0.5% serum, NIH-3T3 cells with vector control cannot proliferate. However, expression of WT or active forms of YAP confers NIH-3T3 cells proliferation potential in low serum medium (Fig. 3*A* and *B*). This is consistent with the oncogenic function of YAP. In contrast, the TBD-defective or WW domain–defective mutants completely lost the ability to promote serum-independent growth. In fact, under low serum conditions, the YAP-S94A–expressing or W1W2-expressing cells displayed a significant decrease in cell numbers, likely due to apoptosis, whereas the vector control cells remain viable (Fig. 3*A* and *B*). However, under normal culture conditions (10% serum), neither YAP-S94A nor YAP-W1W2 expression induced cell death. These results show that both the TBD and WW domains are essential for YAP to promote self-sufficiency of growth signals in NIH-3T3 cells.

**The transformation potential of YAP is inhibited by phosphorylation of serine 127 or 381.** *yap* is a candidate human oncogene amplified in multiple cancers or cancer cell lines (18–21). Elevated YAP expression and nuclear localization is also observed in human cancers (Supplementary Fig. S1*A*). To further establish the function of WW domains in the oncogenic potential of YAP, we first tested if YAP could transform NIH-3T3 cells. Surprisingly, expression of WT YAP does not induce a transforming morphology (Fig. 4*A*). We have previously shown that Lats phosphorylates YAP to inhibit its transactivation and growth promotion activity (14). It is possible that YAP oncogenic potential is also inhibited by Lats-dependent phosphorylation. Mutation of all five serine residues (61, 109, 127, 164, and 381) matching Lats phosphorylation target consensus (HXRXXS) to alanines (YAP-5SA) was reported to make YAP resistant to inhibition by Lats (14). Interestingly, YAP-5SA not only is more potent in stimulating cell proliferation but also causes transformation properties in NIH-3T3 cells (Figs. 3*B* and 4*A*), such as growing on top of each other, indicating the loss of contact inhibition.

We further performed colony formation assays, which are well established to examine oncogenic potential. As expected, YAP-5SA could potently induce colony formation, whereas YAP WT could not (Fig. 4*B* and *C*), which indicates that the oncogenic activity of YAP is inhibited by phosphorylation on at least some of the five sites. However, it is not clear which ones of the five possible sites are critical. To answer this question, we restored individual serine in the YAP-5SA mutant, resulting in YAP-4SA proteins retaining a single putative phosphorylation site. Restoration of serine 127 (4SA/S127) and 381 (4SA/S381) abolished the oncogenic potential

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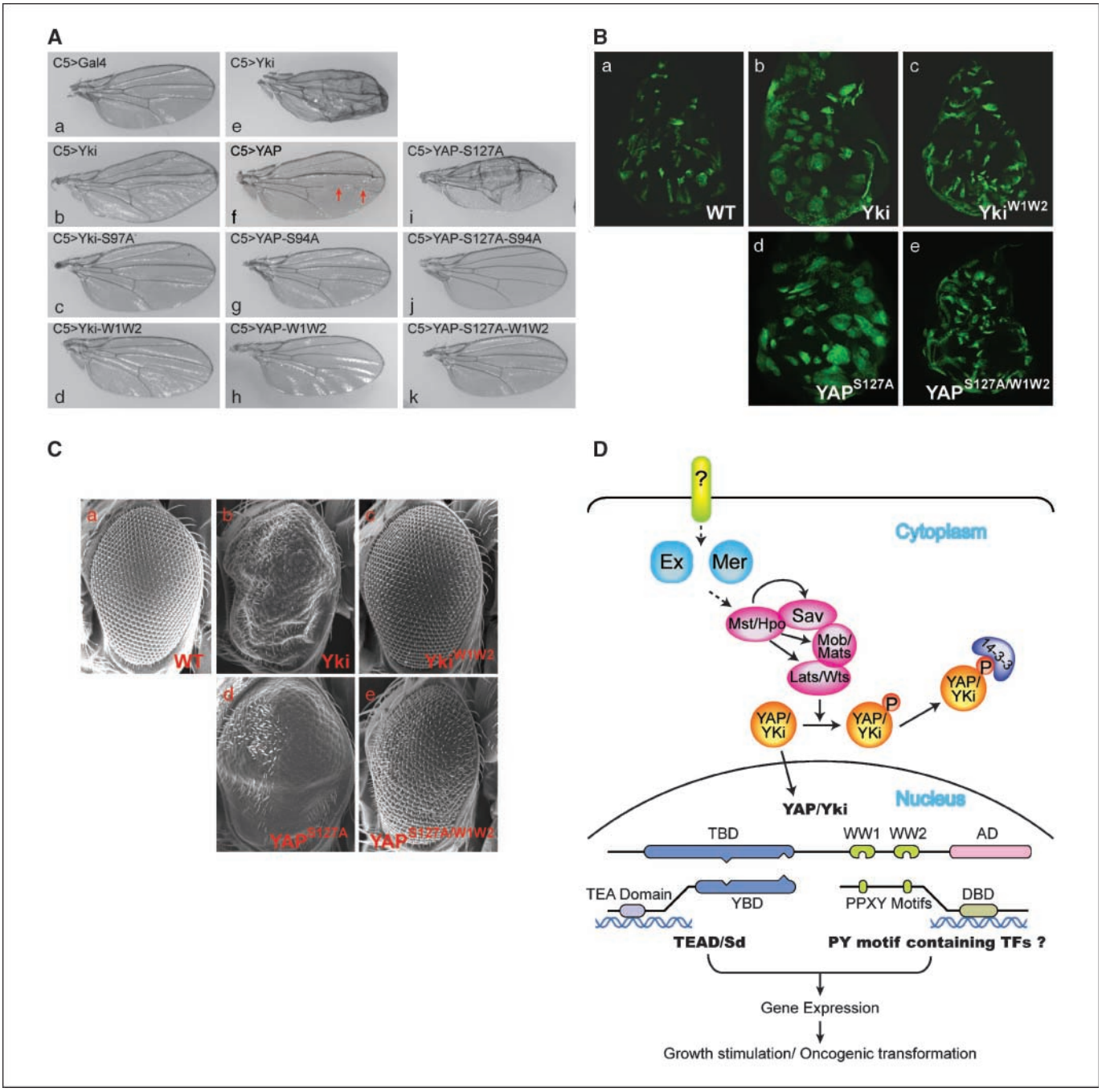


**Figure 6.** The WW domain plays a critical role in YAP/Yki-induced tissue growth. *A,* the TBD and WW domain mutants of YAP/Yki are compromised in promoting wing tissue growth. Overexpression of various *yki* and *yap* transgenes was driven by C5-Gal4. Genotypes of the fly tissues are indicated. *f, arrows,* two gaps along the fourth longitudinal vein. *B,* WW domain mutants of Yki and YAP are compromised in inducing clone expansion. Wing imaginal discs containing 72-h-old control (*a*) or various YAP/Yki-overexpressing clones (*b-e*) were generated by flip-out and positively marked by GFP. Genotypes of the fly tissues are *hsFLP/+; act> y+>Gal4, UAS-GFP$^{S65T}$/+* (*a*), *hsFLP/+; act>y+>Gal4, UAS-GFP$^{S65T}$/UAS-yki-V5* (*b*), *hsFLP/+; act>y+>Gal4, UAS-GFP$^{S65T}$/UAS-yki$^{W1W2}$-V5* (*c*), *hsFLP/+; act>y+>Gal4, UAS-GFP$^{S65T}$/UAS-Flag-YAP$^{S127A}$* (*d*), and *hsFLP/+; act>y+>Gal4, UAS-GFP$^{S65T}$/UAS-Flag-YAP$^{S127A/W1W2}$* (*e*). *C,* the WW domains are important for Yki- and YAP-induced increase of eye size and disruption of retinal patterning. Genotypes of the fly tissues are WT (Canton S; *a*), *GMR-Gal4/UAS-yki-V5* (*b*), *GMR-Gal4/UAS-yki$^{W1W2}$-V5* (*c*), *GMR-Gal4/UAS-Flag-YAP$^{S127A}$* (*d*), and *GMR-Gal4/UAS-Flag-YAP$^{S127A/W1W2}$* (*e*). *D,* proposed model of YAP/Yki transcription factor interaction under negative regulation by the Hippo pathway. YAP/Yki interacts with TEAD family transcription factors through the TBD, and with PPXY motif-containing transcription factors through the WW domains. By these two folds, YAP/Yki activates gene expression, and therefore stimulates growth and promotes oncogenic transformation. *YBD,* YAP-binding domain; *DBD,* DNA-binding domain; *AD,* activation domain. For the Hippo pathway components, their names in both mammals and *Drosophila* are given if different. *Dashed arrows,* unknown biochemical mechanisms.

of YAP-5SA. In contrast, restoration of serine residues 109 (4SA/S109) and 164 (4SA/S164) did not abolish the transforming activity of YAP-5SA/S61. Although YAP-4SA/S61 transfected cells did not form any colony, we could not conclude the importance of S61 due to lack of expression from this DNA construct. These data suggest that phosphorylation of serine 127 or 381 is sufficient to inhibit YAP, therefore abolishing its transformation activity. Consistently, although phosphorylation of serine 127 is known to mediate YAP

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inhibition, YAP-S127A single site mutant is not able to transform NIH-3T3 cells (Fig. 4*C*).

S127 of YAP is directly phosphorylated by Lats (14). We performed *in vitro* kinase assay to test if S381 is also a direct Lats target site. Lats could potently phosphorylate WT YAP but has little activity toward YAP-5SA (Fig. 4*D*). All YAP-4SA mutants could be phosphorylated by Lats with varying efficiency. These data suggest that all five Lats target consensus phosphorylation sites could be phosphorylated by Lats at least *in vitro*.

Using the available phosphorylated YAP S127 antibody, we compared YAP phosphorylation in several cell lines. Among them, MCF10A, a noncancerous cell line, showed the highest phosphorylation level, and ACHN, a cancer cell line showing loss of contact inhibition, has very little YAP phosphorylation (Supplementary Fig. S1*B*). The impaired YAP phosphorylation in ACHN is likely due to mutation of Sav, a key component of the Hippo pathway (41). Collectively, YAP is capable of transforming NIH-3T3 cells, which is inhibited by phosphorylation on the Hippo pathway target sites, and dysregulation of YAP phosphorylation is observed in cancer cells.

**Both the TBD and WW domains are important for the oncogenic activity of YAP.** How YAP activates gene expression to promote oncogenesis is not clear. Based on the ability of YAP-5SA to transform NIH-3T3 cells, we tested the role of the TBD and WW domains, two domains mediating YAP-transcription factor interactions, in YAP-induced oncogenic transformation. Either the TBD or the WW domain was mutated in YAP-5SA, and their transformation activity was examined. As expected, WT, S94A, and W1W2 mutant YAP could not transform NIH-3T3 cells (Fig. 5*A* and *B*). However, in the YAP-5SA background, mutation of either the TBD or WW domains significantly decreased the number of colonies induced, indicating the importance of both domains in the oncogenic transformation activity of YAP.

**TEAD/Sd-binding and WW domains are important for YAP/Yki to promote tissue growth in *Drosophila*.** To examine the significance of the TBD and WW domains in YAP-induced tissue growth, we generated transgenic flies that express human YAP, YAP-S94A, YAP-W1W2, YAP-S127A, YAP-S127A/S94A, or YAP-S127A/W1W2 in developing wings. Similar constructs derived from fly Yki were also used for *in vivo* functional analysis. Expression of human YAP during *Drosophila* wing development increased the wing size by 14% (Fig. 6*A, a* and *f*; S2*A*). Morphology of 4% of the YAP-expressing wings was severely disrupted, and therefore, such flies were not included for wing size analysis. However, overexpression of YAP-S94A or YAP-W1W2 did not show significant change of wing size compared with the control flies (Fig. 6*A, g* and *h*; Supplementary Fig. S2*A*). In addition to the increase of wing size, YAP caused patterning defect of the wings, with the fourth longitudinal vein broken into three segments (Fig. 6*A, f*). This phenotype was not observed in YAP-S94A or YAP-W1W2 flies (Fig. 6*A, g* and *h*). As expected, active YAP-S127A was highly potent to cause severe malformation of the wing with large air bubbles in between apical and basal layers, which made it impossible to correctly measure the wing size (Fig. 6*A, i*). Mutation of S94A or W1W2 dramatically decreased the activity of YAP-S127A, so that the size and morphology of their wings was similar to that of control flies (Fig. 6*A, j* and *k*; Supplementary Fig. S2*A*). In case of fly Yki, its overexpression significantly increased the wing size by 27% (Fig. 6*A, b*; Supplementary Fig. S2*A*) and ~80% of the wings were too malformed to be measured correctly (Fig. 6*A, e*). Both S97A and W1W2 mutations reduced Yki activity, as wings of Yki-S97A and Yki-W1W2 flies were only 19% and 7% larger than WT controls (Fig. 6*A, c* and *d*; Supplementary Fig. S2*A*). Thus, both TEAD/Sd-binding and WW domains are critical for YAP and Yki proteins to promote tissue growth and control organ size.

The functional significance of Yki and YAP WW domains was further investigated in two additional assays. First, Yki/YAP and their derivatives were clonally expressed and their ability to promote clone expansion in wing discs was monitored. Compared with WT controls, both Yki and YAP-S127A strongly stimulated clone expansion so that individual clones as well as the entire wing discs were larger (Fig. 6*B, a, b*, and *d*). However, mutations in WW domains greatly reduced the activity of Yki and YAP-S127A as both the average clone size and wing disc size are similar to those of WT controls (Fig. 6*B, a–e*). In the second assay, both Yki-W1W2 and YAP-S127A/W1W2 were much less potent in increasing the adult eye size and disrupting retinal patterning (Fig. 6*C, a–e*). As expected, they were also less potent than Yki and YAP-S127A, respectively, in increasing the number of interommatidial cells (Supplementary Fig. S2*B, a–e*). These results further support our hypothesis that WW domains are important for the growth-promoting activity of Yki and YAP.

## Discussion

YAP is a candidate oncogene that also regulates organ size. However, the mechanism by which YAP regulates oncogenesis and organ size is not well understood. Recent studies have shown that the TEAD family transcription factors play a critical role in mediating YAP-dependent gene induction, growth promotion, and transformation (27). However, we also observed that a subset of YAP target genes could be induced by the TEAD-binding–defective YAP-S94A mutant (27). Furthermore, *Drosophila* genetics study also showed that *yki* mutant cells have more severe growth defects than *sd* mutant cells (11, 29, 30), and overexpression of the Sd-binding–defective Yki-S97A elicits a reduced but still obvious overgrowth in *Drosophila* eyes and wings (27). These observations suggest that there are other transcription factors mediating YAP-induced gene expression and biological functions. WW domains are the most obvious candidate to mediate interactions with other transcription factors. In this study, we established the functional importance of YAP/Yki WW domains in gene expression induction, growth promotion, and oncogenic transformation.

The WW domain of YAP has been suggested to interact with Lats (15, 37), which phosphorylates and inhibits YAP. However, our study suggests a positive role of YAP WW domains in stimulating cell proliferation and oncogenic transformation *in vitro* and to promote tissue overgrowth *in vivo*. We showed that WW domains are not required for YAP inhibition by Lats. Furthermore, the PPXY motif of Lats is also dispensable for YAP inhibition. Although recent articles have documented the importance of WW domain in YAP and PPXY motif in Lats for their interaction, the authors also noticed that YAP fragments without the WW domain could still be phosphorylated by Lats (15), which is consistent with our observation that the WW domain is not required for YAP inhibition by Lats.

We characterized the oncogenic activity of YAP. YAP expression is elevated in several human cancers as shown by human cancer tissue microarray staining. Expression of WT YAP enhances proliferation rate and confers serum-independent growth in NIH-3T3 cells. The phosphorylation-defective YAP-5SA, but not the WT YAP, potently transforms NIH-3T3 fibroblasts. These data support

Published OnlineFirst January 13, 2009; DOI:10.1158/0008-5472.CAN-08-2997



YAP as an oncogene negatively regulated by phosphorylation. Furthermore, mutation of either the TBD or WW domains significantly attenuates the transformation potential of YAP and largely represses YAP/Yki-induced tissue overgrowth in *Drosophila*. Together, as shown in Fig. 6*D*, we propose that under negative regulation by the Hippo pathway, YAP/Yki interacts with TEAD and PPXY motif-containing transcription factors through the TBD and WW domains, respectively, to induce gene expression that leads to growth stimulation and oncogenic transformation.

Several transcription factors, such as ErbB4 cytoplasmic domain, RUNX2, and p73, have been reported to interact with YAP through the WW domain (33–35), although their biological significance was not clear. p73, a p53 family protein, has growth-inhibitory and apoptotic functions, therefore is unlikely to mediate the growth-promoting and oncogenic function of YAP. Knockdown of ErbB4 does not affect proliferation of ACHN cells.[4] All three RUNX family members have a conserved PPXY motif (34). Efforts to simultaneously knock down these three proteins were unsuccessful (data not shown). There are actually more PPXY motif-containing transcription factors in the human genome, which could be potential YAP targets. Future studies are in need to identify the critical target transcription factors that interact with the WW domain of YAP to mediate its function.

It is worth noting that YAP-S94A or YAP-W1W2 mutant not just fails to support serum-independent growth but rather promotes cell death in low serum condition. In contrast, neither of them induces cell death in medium supplemented with 10% serum. There are two possible explanations. First, expression of YAP-S94A or W1W2 imposes a dominant-negative effect on the expression of some YAP target genes important for serum-independent growth. Expression of such a gene is likely to require both the TBD and WW domains. For example, decreased expression of PIK3C2B was seen by expression of either YAP-S94A or W1W2 (Fig. 1*C*). Second, it is also possible that an imbalanced induction of the TBD-dependent or WW domain–dependent YAP target genes induces apoptosis in low serum condition.

Besides charactering the YAP transcription factor interaction domains, this report further clarifies the importance of the five possible Lats phosphorylation sites on YAP in regulation of its transformation potential. Using YAP-4SA proteins retaining a single HXRXXS site, we found that YAP transformation potential is inhibited if serine 127 or 381 is intact. This result suggests that phosphorylation on either one of these residues is sufficient to inhibit the oncogenic activity of YAP, and decreased YAP phosphorylation is observed in ACHN cancer cell line. Phosphorylation of S127 by Lats creates a 14-3-3 binding site to induce YAP cytoplasmic translocation (14). However, the mechanism by which phosphorylation of S381 inhibits YAP requires further study.

The Hippo-YAP pathway is a new connection between control of organ size and cancer. Elucidation of the mechanism of YAP-induced gene expression, growth promotion, and oncogenic transformation is of immediate importance. In this study, we established the function of YAP WW domains in these processes, which might be a new target of pharmacologic intervention in treating human cancer.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

Received 8/6/2008; revised 9/29/2008; accepted 10/1/2008.

**Grant support:** NIH (K-L. Guan) and National Science Foundation (Z-C. Lai). B. Zhao is supported by the University of Michigan Rackham Predoctoral Fellowship.

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We thank Dr. Marius Sudol for pCMV-Flag-YAP2 and pM-ErbB4-CTF$\Delta$K constructs, Dr. Tian Xu for Lats2, Dr. Duojia Pan for pGal4-yki, Dr. Georg Halder for *hsFLP; act>y+>GFP-S65T*, Dr. Hongjiao Ouyang for RUNX2 and 6× OSE2-luc reporter, Dr. Ben Margolis for MCF10A cells, the Developmental Studies Hybridoma Bank at the University of Iowa for Dlg antibody, Dr. Xiaomu Wei for generating UAS-Flag-YAP$^{S94A}$ and UAS-Flag-YAP$^{W1W2}$ transgenic flies, Dr. Taocong Jin for assistance on gene expression microarray, and Karen Tumaneng and Kristen Slanina for critical reading of the manuscript.

[4] Unpublished observation.

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Published OnlineFirst January 13, 2009; DOI:10.1158/0008-5472.CAN-08-2997



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