Transcriptional Output of the Salvador/Warts/Hippo Pathway Is Controlled in Distinct Fashions in Drosophila melanogaster and Mammalian Cell Lines

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Abstract

The Salvador/Warts/Hippo (SWH) pathway is an important modulator of organ size, and deregulation of pathway activity can lead to cancer. Several SWH pathway components are mutated or expressed at altered levels in different human tumors including NF2, LATS1, LATS2, SAV1, and YAP. The SWH pathway regulates tissue growth by restricting the activity of the transcriptional coactivator protein known as Yorkie (Yki) in Drosophila melanogaster and Yes-associated protein (YAP) in mammals. Yki/YAP drives tissue growth in partnership with the Scalloped (Sd)/TEAD1-4 transcription factors. Yki/YAP also possesses two WW domains, which contact several proteins that have been suggested to either promote or inhibit the ability of Yki to induce transcription. To investigate the regulatory role of the Yki/YAP WW domains, we analyzed the functional consequence of mutating these domains. WW domain mutant YAP promoted transformation and migration of breast epithelial cells with increased potency, suggesting that WW domains mediate the inhibitory regulation of YAP in these cells. By contrast, the WW domains were required for YAP to promote NIH-3T3 cell transformation and for the ability of Yki to drive tissue growth in D. melanogaster and optimally activate Sd. This shows that Yki/YAP WW domains have distinct regulatory roles in different cell types and implies the existence of proteins that promote tissue growth in collaboration with Yki and Sd.

Introduction

The Salvador/Warts/Hippo (SWH) tumor suppressor pathway is a critical mediator of organ size in Drosophila melanogaster and mammals (1–3). Upstream regulators of the SWH pathway include the atypical cadherins Fat and Dachsous and the Band 4.1 proteins Expanded (Ex) and Merlin (4–9). In an as yet unidentified fashion, these proteins control activity of the core SWH pathway proteins: the S/T kinases Hippo (Hpo) and Warts (Wts) and the adaptor proteins Salvador (Sav) and Mob as tumor suppressor (Mats; refs. 10–18). These proteins restrict tissue growth by limiting activity of the Yorkie (Yki) transcriptional coactivator protein [homologous to Yes-associated protein (YAP) and TAZ (WWTR1)] in mammals; ref. 12].

More recently, the SWH pathway has been shown to be functionally conserved in mammals as is its basic mechanism of signal transduction (19–22). Deregulation of SWH pathway activity has been implicated in the genesis of multiple human cancers (1–3). Several SWH pathway tumor suppressor genes are mutated or show reduced expression in mammalian cancers: NF2 (merlin) is mutated in neurofibromatosis type II (1); the mammalian homologues of sav (SAV1) and mats (MOBK1) are mutated in different human and mouse cancer cell lines (11, 15); expression of the wts homologues LATS1 and LATS2 is silenced in aggressive human breast cancers, whereas LATS1 nullizygous mice develop tumors (1); and reduced expression and deletion of human homologues of fat occurs in breast, oral, and astrocytic cancers (23–25). In addition, several studies have reported oncogenic roles for the yki homologues YAP and TAZ: the YAP gene was amplified in mouse models of breast and liver cancer, and YAP overexpression in mouse liver and gastrointestinal tract induced hyperplasia (19, 20, 26, 27). In addition, YAP protein expression is elevated and nuclear in a high frequency of different human cancers including ovarian, liver, and prostate (19, 21), whereas TAZ expression was elevated in a percentage of invasive human breast tumors (28).

Yki and YAP stimulate gene expression by promoting activity of their cognate transcription factors. The growth-promoting activity of Yki is largely mediated by the Scalloped (Sd) transcription factor (29–31). An analogous interaction in mammals between YAP and the Sd homologues, TEAD1-4, is required for the ability of YAP to transform cultured cells (32, 33). Importantly, several pieces of evidence suggest that Yki/YAP requires proteins other than Sd/TEAD to mediate its growth-promoting activity. Clones of tissue lacking yki grow poorly in all D. melanogaster imaginal disc tissues, whereas tissue lacking sd only displays a growth deficiency in the pouch region of the developing wing imaginal disc but not in other tissues such as eye or antenna (12, 29). In addition, a version of human YAP harboring a mutation that aborts interaction with Sd was still capable of promoting tissue growth when misexpressed in the D. melanogaster eye (32).

Yki/YAP interacts with Sd/TEAD via a conserved region in the amino terminus of Yki/YAP (29, 30, 32, 34). YAP WW domains also mediate interaction with transcription factors including pEBP2α, ErbB-4, and p73 by contacting PPxY (PY) motifs in these proteins (35–39). YAP can activate these transcription factors in vitro, but the in vivo biological function of these interactions has not been explored. Interestingly, the WW domains of Yki and YAP also mediate interaction with the SWH pathway kinase, Wts (LATS1/2 in mammals), which serves to inhibit Yki/YAP activity by phosphorylation-dependent inhibition of several key S residues, most prominently Yki-S168, and YAP-S127 (19, 21, 22, 40). Therefore, Yki/ YAP WW domains potentially interact with proteins that either promote or inhibit its ability to stimulate tissue growth.
To investigate the regulatory roles of the Yki/YAP WW domains, we conducted a series of experiments in D. melanogaster and human tissue culture cells. The ability of YAP to transform MCF10A human breast epithelial cells was enhanced when the WW domains were mutated, suggesting that the primary role of these domains in MCF10A cells is to inhibit YAP. Strikingly, an opposing role was observed for Yki/YAP WW domains in NIH-3T3 cells and D. melanogaster; WW domains were essential for the ability of Yki/YAP to stimulate transformation and tissue growth, respectively. This suggests that SWH pathway activity is regulated in different cell types by distinct modes at the level of the Yki/YAP transcriptional coactivator proteins.

Materials and Methods

D. melanogaster stocks. D. melanogaster stocks were generated harboring the following transgenes: UAS-Yki-YFP, UAS-Yki-YFP-S168A, UAS-Yki-YFP-WW1*2*, UAS-Yki, UAS-Yki-S168A, or UAS-Yki-WW1*2*. Misexpression and clonal experiments were conducted using the above stocks and GMR-Gal4, engrailed-Gal4, hsFLP UAS:CD8GFp; FRT42D tab; Gal80/FRT42D; tub; Gal4, w; FRT42D; or w; FRT42D yki. 

Immunohistochemistry. Tissues were stained as described (41) using DIAP1 (B. Hay), Ex (A. Laughon), and secondary antibodies (Invitrogen).

Expression plasmids. Mutations were introduced into pAc5.1-Yki-HA, pAc5.1-V5-Wts (D. Pan), or pBabe-FLAG-YAP (D. Haber) as detailed (Supplementary Data). pQCXIH-YAP constructs were kindly provided by K. Guan. Transgene constructs were cloned into pTWV (T. Murphy) or pUAST-AttB.

Luciferase assays. D. melanogaster S2 cells were transfected with plasmids expressing wild-type or mutant Yki, pCaSper-hsp-Gal4db-SD (A. Zider), pUAST-LUC, and pGL3-HSP-Renilla (internal control). HEK293 cells were transfected with plasmids expressing wild-type or mutant YAP. pcDNA3.0-TEAD2, pGT4T-LUC (M. DePamphilis), and pRL-CMV (internal control). HEK293 cells were transfected with plasmids expressing wild-type or mutant Yki, pCaSper-hsp-Gal4db-SD (A. Zider), pUAST-LUC, and pGL3-HSP-Renilla (internal control). HEK293 cells were transfected with plasmids expressing wild-type or mutant YAP. pcDNA3.0-TEAD2, pGT4T-LUC (M. DePamphilis), and pRL-CMV (internal control). HEK293 cells were transfected with plasmids expressing wild-type or mutant Yki, pCaSper-hsp-Gal4db-SD (A. Zider), pUAST-LUC, and pGL3-HSP-Renilla (internal control). HEK293 cells were transfected with plasmids expressing wild-type or mutant YAP. pcDNA3.0-TEAD2, pGT4T-LUC (M. DePamphilis), and pRL-CMV (internal control). HEK293 cells were transfected with plasmids expressing wild-type or mutant Yki, pCaSper-hsp-Gal4db-SD (A. Zider), pUAST-LUC, and pGL3-HSP-Renilla (internal control). HEK293 cells were transfected with plasmids expressing wild-type or mutant YAP. pcDNA3.0-TEAD2, pGT4T-LUC (M. DePamphilis), and pRL-CMV (internal control). HEK293 cells were transfected with plasmids expressing wild-type or mutant Yki, pCaSper-hsp-Gal4db-SD (A. Zider), pUAST-LUC, and pGL3-HSP-Renilla (internal control). HEK293 cells were transfected with plasmids expressing wild-type or mutant YAP. pcDNA3.0-TEAD2, pGT4T-LUC (M. DePamphilis), and pRL-CMV (internal control).

Immunoprecipitations and immunoblotting. S2 cells transfected with plasmids encoding wild-type or mutant Yki and Wts proteins were lysed after 48 h and immunoprecipitated with rabbit anti-HA (Sigma). Immunoprecipitations were subjected to SDS-PAGE and immunoblotted with mouse anti-V5 (Invitrogen) or rat anti-HA (Roche). Third instar larval eye discs were dissected, lysed, immunoprecipitated with anti-Myc 9E10, and immunoblotted with anti-Yki (K. Irvine) or anti-Myc 9E10. Other antibodies detected YAP (Cell Signaling), tubulin (Sigma), proliferating cell nuclear antigen (BD Transduction), human actin (MP Biomedicals), and D. melanogaster actin (DSHB).

Mammalian cell culture and analysis. Infection of MCF10A and NIH-3T3 cells, two- and three-dimensional MCF10A culture, soft agar, and cell proliferation assays were done as described previously (42, 43). Cell migration assays were done as described previously (44). Cell fractionation was done using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology).

Results

Multiple Yki WW domains and Wts PY motifs are required for efficient interaction between Yki and Wts. Yki possesses two WW domains, whereas different isoforms of YAP possess one or two WW domains (45). To investigate the function of Yki/YAP WW domains, we mutated the second conserved W to F and the conserved P to A. This mutation strategy abolishes the binding capacity of WW domains without grossly altering its structure (46, 47). Initially, we sought to determine which Yki WW domains were required to interact with Wts and which Wts PY motifs mediated interaction with Yki. D. melanogaster S2 cells were transfected with plasmids encoding wild-type or mutant versions of HA-tagged Yki and V5-tagged Wts. Cell lysates were immunoprecipitated with either anti-V5 or anti-HA, subjected to SDS-PAGE, and immunoblotted with the appropriate antibodies. A specific interaction between Wts and either Yki WW domains was detected in vivo in developing D. melanogaster eye discs (Fig. 1A, B). This was confirmed in vitro in developing D. melanogaster eye discs where Yki, but not Yki-WW1*2*, associated with Wts (Fig. 1B). These results differ slightly from those of YAP, which mediates interaction with the mammalian Wts orthologue, LATS1, predominantly via WW domain 1 (22).

To identify which Wts PY motifs were important for contacting Yki, we mutated each Wts PY motif individually by converting the conserved Y to A, which renders PY motifs incapable of binding to WW domains (48). We observed no substantial effect on interaction between Wts and Yki when Wts PY motifs were mutated individually, suggesting that Yki can contact multiple Wts PY motifs (data not shown). Next, we repeated immunoprecipitations...

Figure 1. Multiple Yki WW domains and Wts PY motifs are required for Yki and Wts to interact efficiently. A, wild-type Yki, Yki-WW1*, Yki-WW2*, and Yki-WW1*2* were assessed for their ability to interact with Wts in S2 cells. Immunoprecipitations were done with rabbit anti-HA antibodies and immunoblots were done with mouse anti-V5 (to detect Wts) or rat anti-HA (to detect Yki). B, Yki-YFP and Yki-YFP-WW1*2* were analyzed for their ability to immunoprecipitate Myc-Wts in developing D. melanogaster eye discs. Immunoprecipitates were done with anti-Myc and immunoblots with anti-Yki or anti-Myc. C, Wts proteins with mutations in either two or three PY motifs were assessed for their ability to bind to Yki. Immunoprecipitations were done as in A. In A to C, immunoblots were done on either immunoprecipitates (IP) or input lysate (input).
using Wts proteins harboring various combinations of mutant PY motifs (1 and 2; 2 and 4; 1, 2, and 3; or 2, 3, and 4). A partial reduction in Wts-Yki binding was observed when Wts PY motifs 1 and 2 or motifs 2 and 4 were mutated (Fig. 1C). No further reduction in binding was observed when PY motifs 1, 2, and 3 were mutated. Wts-Yki binding was substantially impeded when PY motifs 2, 3, and 4 were abolished with only minimal interaction observed (Fig. 1C). Collectively, immunoprecipitation studies suggest that, for efficient binding to Wts, both Yki WW domains must contact a Wts PY motif and that Yki can interact with different Wts PY motifs interchangeably, although Yki displays some preference for PY motif 4.

**Mutation of YAP WW domains promotes invasive growth of MCF10A cells.** To examine the function of YAP WW domains, we generated a series of MCF10A cell lines (immortalized human breast epithelial cells) expressing wild-type human YAP or various YAP mutants YAP with mutations in WW domain 1 (YAP-WW1*); WW domain 2 (YAP-WW2*); both WW domains (YAP-WW1*2*); or a hyperactive version of YAP carrying a S-to-A mutation at residue 127 (YAP-S127A), which is a major site of inhibitory phosphorylation mediated by the LATS1/2 kinase (Fig. 2A; refs. 19, 21, 22).

To determine whether YAP WW domains mediate positive or negative regulatory interactions, we assessed the ability of wild-type and mutant YAP proteins to promote MCF10A cell transformation and migration. MCF10A cells grown in three-dimensional Matrigel culture form polarized acini, but when overexpressing YAP, acini form structures with spike-like protrusions, characterized as an invasive phenotype (Fig. 2A and B; refs. 21, 27). Consistent with previous findings, we found that YAP-S127A was more potent than wild-type YAP at stimulating invasive MCF10A acini. When YAP-WW1* or YAP-WW2* were overexpressed in MCF10A cells, a significant increase in invasive acini was recorded, and when both WW domains were mutated, YAP overexpression stimulated even more invasive acini (Fig. 2B).

**WW domain mutations enhance the ability of YAP to promote anchorage-independent cell growth.** The ability to confer anchorage-independent cell growth is a hallmark of oncogenes such as Ras, PI3-kinase and YAP (27). Consistent with previous reports, we found that YAP promoted anchorage-independent growth of MCF10A cells in soft agar, and YAP-S127A did so with increased potency. In addition, increased numbers of colonies were observed in cells expressing either YAP-WW1* or YAP-WW2*. In accordance with results from our Matrigel assay, an
even greater increase in colony formation was observed when MCF10A cells overexpressed YAP-WW1*2* (Fig. 2C).

Mutation of YAP WW domains promotes MCF10A cell migration. To assess the effect of YAP mutations on cell migration, scratch assays were done on confluent MCF10A cells. After a scratch was done, cells were incubated in the presence of the proliferation inhibitor, mitomycin C, and wound closure was observed over 24 h. Results were highlighted by dashed white lines (indicating the invasive front) and quantitated (Supplementary Fig. S1). YAP overexpression increased the rate of wound closure compared with control cells, whereas YAP-S127A, YAP-WW1*, YAP-WW2*, or YAP-WW1*2* overexpression further increased the rate of wound closure (Fig. 2D). This suggests that, like S127A, WW domain mutations confer gain-of-function properties with respect to the ability of YAP to enhance cell migration. Collectively, results in Fig. 2 show that YAP WW domains normally inhibit its ability to stimulate transformation and migration of MCF10A cells and suggest that the primary function of the WW domains of YAP is to mediate contact with an inhibitory protein(s). In addition, our data suggest that transcription factors such as pEBP2α, p73, and Erb-B4, which bind to YAP WW domains and are activated by YAP in vitro, do not have a major influence on the ability of YAP to transform MCF10A cells or stimulate their migration.

YAP WW domains have cell-specific regulatory functions. While this paper was under review, YAP WW domains were shown to have a seemingly different role with respect to cell transformation than reported here. A highly active version of YAP that has five LATS1/2 phosphorylation sites removed (YAP-5SA) was shown to transform murine NIH-3T3 cells with reduced potency when the WW domains were mutated (43). We considered two possibilities to explain this apparent discrepancy: (a) WW domains have different regulatory functions in MCF10A and NIH-3T3 cells. (b) As YAP-5SA is impervious to SWH pathway-mediated repression, it is maximally activated; therefore, mutation of the WW domains, which contact LATS1/2, cannot increase YAP activity further. To test these hypotheses, we assessed the ability of the various YAP proteins we generated to promote transformation and proliferation of NIH-3T3 cells. In contrast to our results in MCF10A cells, we

Figure 3. YAP WW domains have cell-specific regulatory functions. Aa, expression levels of endogenous mouse YAP (Con) and overexpressed human wild-type and mutant YAP proteins in stably infected NIH-3T3 cells. Ab, quantitation of colonies expressing the indicated plasmids grown in soft agar. Ac, proliferation curves of NIH-3T3 cells expressing the indicated plasmids over 7 d. Bb, expression levels of endogenous YAP (Con) and overexpressed wild-type and mutant YAP proteins in stably infected MCF10A cells. Bb, quantitation of MCF10A colonies expressing the indicated plasmids grown in soft agar. Bc, MCF10A wound closure assays done over 24 h on cells expressing the indicated plasmids. Bars, SD (n = 3; Ab, Ac, Bb, and Bc).
found that WW domain mutant YAP had reduced capability to induce anchorage-independent growth of NIH-3T3 cells (Fig. 3Ab). In addition, we found that YAP-WW1*2* enhanced the proliferation rate of NIH-3T3 cells with less potency than either YAP or YAP-S127A, consistent with previous observations (Fig. 3Ac; refs. 33, 43). These results show that YAP WW domains indeed have cell-specific regulatory roles.

To assess a potential positive regulatory role for YAP WW domains in MCF10A cells, we generated stably infected cells expressing wild-type YAP, YAP-5SA, or YAP-5SA with either the TEAD binding domain or WW domains mutated (43). Consistently, we found that YAP-5SA and YAP-5SA-WW1*2* were expressed at higher levels than YAP or YAP-5SA-S94A (Fig. 3Bb). YAP-5SA stimulated anchorage-independent growth (Fig. 3Bb) and migration in scratch assays (Fig. 3Bc) with greater potency than wild-type YAP. Interestingly, YAP-5SA-WW1*2* induced slightly more soft-agar colonies than YAP-5SA, suggesting that WW domains interact with an inhibitory protein(s) in addition to LATS1/2 (Fig. 3Bb). By contrast, when the WW domains were mutated, YAP-5SA activity was partially abrogated with respect to cell migration, suggesting that YAP WW domains interact with a positive regulatory protein(s) that modulates MCF10A cell-migratory behavior and that this regulation is only uncovered when assessing activity of maximally activated YAP (Fig. 3Bc).

Mutating the WW domains of YAP does not affect its subcellular localization. Given that LATS1/2 interact with YAP WW domains and cause cytoplasmic sequestration, we reasoned that WW mutations might promote the activity of YAP by causing nuclear accumulation. Therefore, we used subcellular fractionation to analyze the localization of YAP, YAP-S127A, and YAP-WW1*2* in MCF10A cells. Wild-type YAP was mostly cytoplasmic and partially nuclear, whereas YAP-S127A was found in both compartments at roughly equivalent levels. By contrast, YAP-WW1*2* was mostly cytoplasmic resembling localization of wild-type YAP (Fig. 4A). This shows that, unlike the S127A mutation, WW domain mutations do not cause nuclear accumulation of YAP, suggesting that abolition of WW domain function has a gain-of-function effect on YAP independent of subcellular localization.

Mutating the WW domains of YAP enhances its ability to activate TEAD transcription factors. YAP stimulates cell transformation and migration in partnership with TEAD1-4 transcription factors (29–33). We therefore determined whether YAP-WW1*2* activated TEADs more potently by performing luciferase-based transcription assays in HEK293 cells. When plasmids expressing TEAD2, YAP, or YAP-WW1*2* were transiently transfected into HEK293 cells, we observed only mild activation of TEAD-specific luciferase activity (Fig. 4B). Similar levels of activity were observed in cells expressing vector alone (control) or a version of YAP harboring WW domain mutations and a S-to-A mutation at residue 94 (YAP-WW1*2*S94A). S94 is essential for YAP to bind TEADs and transform MCF10A cells (32). When YAP and TEAD2 were coexpressed in HEK293 cells, robust luciferase activity was recorded (~23-fold higher than control). When TEAD2 was coexpressed with either YAP-WW1*2* or YAP-S127A, a further increase in luciferase activity was observed (30- and 32-fold higher than control, respectively). To determine the specificity of activation of TEAD-dependent transcription by YAP-WW1*2*, we measured luciferase activity in the presence of TEAD2 and YAP-WW1*2*S94A. In this scenario, activation of TEAD was abolished (Fig. 4B).

Given that YAP-WW1*2* robustly activated TEAD2, we investigated whether the gain-of-function properties of YAP-WW1*2* were reliant on TEADs by generating MCF10A cells expressing YAP-WW1*2*S94A as in Fig. 2. Consistent with our previous results, YAP WW domain mutations caused a significant increase in the ability of YAP to promote anchorage-independent growth, invasive growth in Matrigel, and cell migration. However, when the TEAD-binding ability of YAP-WW1*2* was removed by introduction of S94A, it failed to promote cell transformation and migration, showing that, like wild-type YAP, YAP-WW1*2* activates TEAD transcription factors to induce MCF10A cell transformation and migration (Supplementary Fig. S2).

**WW domains are required for Yki to promote growth of D. melanogaster tissues.** Once we had determined a regulatory role for YAP WW domains in cell culture, we sought to validate these results in vivo by analyzing the effect of WW mutations on the YAP orthologue, Yki, in *D. melanogaster*, which stimulates growth of epithelial imaginal discs. We generated transgenic *D. melanogaster* harboring different Yki proteins fused to yellow fluorescent protein (YFP): wild-type Yki, gain-of-function Yki (Yki-YFP-S168A), or Yki with both WW domains mutated (Yki-YFP-WW1*2*). When Yki-YFP was misexpressed under control of the *glass multimeric* reporter (*GMR*; two copies of each transgene), overgrowth of the adult eye...
resulted (Fig. 5A, b and f). When Yki-YFP-S168A was misexpressed with GMR-Gal4 (one copy of each transgene), we also observed strong eye overgrowth (Fig. 5A, c and g). By contrast, misexpression of two copies of Yki-YFP-WW1*2* with two copies of GMR-Gal4 did not stimulate any tissue overgrowth, with these eyes resembling the size and rough appearance of flies expressing two copies of GMR-Gal4 alone (compare Fig. 5A, a, e and d, and h). The growth-promoting properties of 6 to 12 independent transgene insertions of each Yki-YFP construct were assessed, and identical results were observed. This indicates that WW domains are required for the ability of Yki to promote tissue overgrowth in D. melanogaster and contrasts with our functional analysis of YAP WW domains, which inhibit the ability of YAP to promote MCF10A cell transformation and migration.

We then examined the ability of different wild-type and mutant Yki-YFP proteins to induce expression of two SWH pathway target genes: DIAP1 and ex. Identical confocal microscope settings were used to image DIAP1 and Ex protein expression in third instar larval eye imaginal discs and consistent results observed in at least 10 discs of each genotype. Elevated expression of DIAP1 and Ex was observed in the GMR expression domain (posterior to the morphogenetic furrow) in eye discs expressing two copies of GMR-Gal4 and Yki-YFP (Fig. 5B, b and f) and in discs expressing GMR-Gal4 and Yki-YFP-S168A (Fig. 5B, c and g). By contrast, discs expressing two copies of GMR-Gal4 and Yki-YFP-WW1*2* showed no induction of DIAP1 or Ex (Fig. 5B, d and h), similar to the negative control (two copies of GMR-Gal4; Fig. 5B, a and e). These data directly correlate with the relative

Figure 5. Yki WW domains are required for its ability to promote tissue growth and Sd-dependent transcription in D. melanogaster. A, side profiles (a-d) and dorsal profiles (e-h) of adult D. melanogaster heads expressing two copies of GMR-Gal4 (a and e) or two copies of GMR-Gal4 and either Yki-YFP (b and f) or Yki-YFP-WW1*2* (d and h) or one copy of GMR-Gal4 and Yki-YFP-S168A (c and g). B, third instar larval eye imaginal discs (anterior is to the right) expressing transgenes described in A and analyzed for DIAP1 protein (a-d) or Ex protein expression (e-h). The domain of GMR-dependent misexpression is posterior to the morphogenetic furrow (arrowheads). C, differential interference contrast images of third instar larval eye imaginal discs (anterior is to the right) harboring wild-type clones (a), yki clones (b), or yki clones expressing either wild-type yki (c), yki-S168A (d), or yki-WW1*2* (e) under control of the UAS promoter. All clones express GFP (green). D, luciferase assay measuring Sd-dependent transcriptional activity in S2 cells expressing either vector (Con), Yki, Yki, Sav, Wts, and Hpo (Yki+SWH), or Yki-WW1*2*. Bars, SD (n = 3). Db, immunoblot showing expression of wild-type and mutant Yki-YFP proteins in S2 cells.
ability of these different Yki-YFP proteins to induce tissue growth (Fig. 5).

To more completely analyze Yki WW domain function, it was necessary to investigate the role of these domains for normal tissue growth by assessing the ability of wild-type and mutant Yki proteins to rescue the growth deficit of tissue lacking endogenous yki. A range of transgenic *D. melanogaster* (UAS-Yki, UAS-Yki-S168A, and UAS-Yki-WW1*2*) was generated with each transgene integrated into an identical genomic locus by using the F capacit integration technique (49). Importantly, this method ensured that each transgene was expressed at identical levels. Clones of tissue of different genotypes were induced using the MARCM technique (50) 48 h after egg deposition and clone size was analyzed 120 h after egg deposition. As described previously, *yki* clones grew poorly and were substantially smaller than wild-type clones (Fig. 5; ref. 12).

The growth of *yki* clones expressing either Yki or Yki-S168A under the control of the UAS promoter was similar to wild-type clones, showing a complete rescue of endogenous *yki*. By contrast, Yki-WW1*2* was unable to rescue the growth deficiency associated with *yki* loss, showing that the WW domains are essential for Yki to promote normal growth and proliferation of imaginal disc epithelial cells (Fig. 5).

**WW domains of Yki promote its ability to activate the Sd transcription factor.** Next, we sought to explore the mechanism by which Yki WW domains promote its ability to drive tissue growth. Given that Yki stimulates tissue overgrowth in conjunction with Sd, we analyzed the result of mutating the WW domains of Yki on its ability to activate Sd using a S2 cell-based luciferase assay (31). Wild-type Yki robustly activated Sd-dependent luciferase activity (~250-fold over vector control; Fig. 5D, a). To determine the specificity of this induction, we cotransfected Yki with plasmids encoding the core SWH pathway proteins, Sav, Wts, and Hpo, which reduced Sd activation by Yki to ~25-fold (10 times less than in their absence). Yki-WW1*2* induced luciferase activity by ~100-fold, roughly 2.5 times less than wild-type Yki, despite the fact that each Yki protein was expressed at comparable levels (Fig. 5D, b). These transcriptional activity results are consistent with our in vivo data (Fig. 5A), showing that Yki WW domains are required to induce tissue overgrowth, which is reliant on Sd.

**WW domains of Yki are required for localization at the apical junction of *D. melanogaster* wing imaginal disc cells.** Given that the WW domains were required for the ability of Yki to induce tissue growth, we reasoned that they might mediate translocation to the nucleus where Yki activates its cognate transcription factors. To explore this hypothesis, we investigated the effect of mutating the WW domains of Yki on its subcellular localization. Consistent with previous reports, we found that wild-type Yki-YFP was predominantly cytoplasmic and barely detectable in the nucleus of *D. melanogaster* wing imaginal disc cells (Fig. 6A), whereas Yki-YFP-S168A was localized in both the nucleus and the cytoplasm although still predominantly cytoplasmic (Fig. 6B). Yki-YFP-WW1*2* displayed a similar localization as wild-type Yki-YFP, indicating that the WW domains do not play a major role in defining whether Yki resides in the nucleus or cytoplasm (Fig. 6C).

Interestingly, we also observed accumulation of Yki-YFP and Yki-YFP-S168A at the apical junction of wing imaginal disc cells (Fig. 6A, d and B, d), where other SWH pathway proteins are known to localize such as Dachsous, Fat, Ex, Merlin, and Wts (4–7, 9). By contrast, Yki-YFP-WW1*2* failed to localize at the apical junction and instead displayed diffuse localization at the apical surface of these cells, above the plane of the nuclei (Fig. 6C, d).

**Discussion**

Here, we present a detailed functional analysis of the WW domains of the orthologous Yki/YAP transcriptional coactivator proteins. Surprisingly, we discovered differing regulatory roles for the WW domains of Yki and YAP. WW domains of Yki are required for its ability to stimulate tissue growth in *D. melanogaster* but appear to mediate a negative regulatory interaction for YAP in human MCF10A cells. Consistent with our *D. melanogaster* data, we and others discovered a positive regulatory role for YAP WW domains in mouse NIH-3T3 cells with respect to the ability of YAP to control cell transformation, proliferation, or apoptosis in low-serum conditions (3; refs. 22, 33, 43). This suggests that the Yki/YAP WW domains have cell-specific and/or context-specific regulatory roles.

The simplest explanation for the finding that WW domain mutations promote the ability of YAP to induce cell transformation and migration is that YAP-WW1*2* can no longer bind the inhibitory kinases, LATS1/2. Our initial mechanistic studies suggest that the regulatory properties of YAP WW domains are more complex, however. Wts/LATS1/2 phosphorylates Yki/YAP, leading to sequestration of Yki/YAP in the cytoplasm, rendering them inactive because they cannot gain access to their cognate transcription factor(s). Accordingly, a version of YAP lacking the major...
LAT1 phosphorylation site (YAP-S127A) was enriched in MCF10A nuclei. By contrast, YAP-WW1*2* displayed similar subcellular localization to wild-type YAP: mostly cytoplasmic and only partly nuclear. Therefore, the precise mechanism by which the WW domains mediate negative inhibition of YAP in MCF10A cells appears to be complex. Our data suggest that YAP-WW1*2* activates TEAD transcription factors with greater potency than YAP or YAP-S127A given that YAP-WW1*2* is not obviously enriched in the nucleus. One possibility is that the YAP WW domains contact an additional repressor to LAT1/2 that restricts the transcriptional coactivator ability of YAP without influencing its localization and is supported by the finding that YAP-SSA-WW1*2* induced anchorage-independent growth more potently than YAP-SSA.

Our finding that WW domains are required for Yki/YAP to promote growth of D. melanogaster tissues and transformation of NIH-3T3 cells suggests that these domains not only play an inhibitory function by binding to Wts/LATS1/2 but also associate with proteins that promote Yki/YAP activity. Given that WW domain mutant-Yki had reduced capability to promote Sd activity, candidate proteins are likely to function in partnership with Yki and Sd to promote tissue growth. It is also conceivable that Yki/YAP functions as a coactivator for transcription factors other than Sd/TEAD1-4 and contacts these proteins via its WW domains. This is an attractive hypothesis given that Yki is generally required for growth of developing D. melanogaster epithelial tissues, whereas Sd is required for growth of select tissues (12, 29).

The Yki WW domains are also important for the subcellular localization of Yki in polarized D. melanogaster epithelial cells (Fig. 6). Although we observed no gross differences in the nuclear/cytoplasmic distribution of Yki and Yki-WW1*2*, Yki-WW1*2* failed to associate at the apical junctional membrane of epithelial cells, whereas a percentage of active Yki proteins did (Fig. 6). One possibility is that Yki associates via its WW domains with proteins that activate it at the apical membrane, thereby stimulating translocation to the nucleus. Clearly, defining the precise mechanism by which the Yki WW domains promote tissue growth requires further investigation.

Deregulation of SWI pathway activity has been implicated in the genesis of several human cancers (1). Therefore, the generation of therapies that target the activity of the oncogenes of this pathway, YAP and TAZ, is an attractive strategy to treat cancers that arise due to altered SWI pathway activity. After studying the regulatory role of Yki WW domains in D. melanogaster and NIH-3T3 cells, it would appear reasonable to develop cancer-suppressing compounds that inhibit YAP WW domain function. However, our contradictory findings on the regulatory role of YAP WW domains in MCF10A cells suggest that such an approach might not be effective at least in breast epithelial tumors. These studies highlight the importance of rigorous mechanistic studies in multiple experimental systems and argue for further investigation of the growth-regulatory roles of YAP WW domains in other cell types and tissues. YAP protein is expressed at elevated levels and is prominently nuclear in multiple primary human cancers (19, 21). In future studies, it will be interesting to determine whether mutations occur in the WW domains of YAP in human cancers that have enhanced YAP activity.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments

Received 12/2/08; revised 5/12/09; accepted 6/4/09; published OnlineFirst 7/7/09.

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We thank P. Burke for embryo injections; A. Ogdin for MCF10A cell expertise, M. Sudol for comments; K. Basler, M. DePamphilis, K. Guan, D. Haber, B. Hay, K. Irvine, A. Laughon, L. Tao, T. Murphy, D. Pan, A. Zider, Developmental Studies Hybridoma Bank and Bloomington Stock Centre for reagents; and the Peter MacCallum Cancer Centre Microscopy Facility. K.F. Harvey holds a career development award from the International Human Frontier Science Program Organization. K.F. Harvey and P.O. Humbert hold career development awards and project grants from the National Health and Medical Research Council of Australia.

References

YAP WW Domains Have Cell-Specific Regulatory Functions

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Xiaomeng Zhang, Claire C. Milton, Patrick O. Humbert, et al.

*Cancer Res* Published OnlineFirst July 7, 2009.

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