The Hippo Signaling Pathway Coordinately Regulates Cell Proliferation and Apoptosis by Inactivating Yorkie, the *Drosophila* Homolog of YAP

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Summary

Coordination between cell proliferation and cell death is essential to maintain homeostasis in multicellular organisms. In *Drosophila*, these two processes are regulated by a pathway involving the Ste20-like kinase Hippo (Hpo) and the NDR family kinase Warts (Wts; also called Lats). Hpo phosphorylates and activates Wts, which in turn, through unknown mechanisms, negatively regulates the transcription of cell-cycle and cell-death regulators such as *cycE* and *diap1*. Here we identify Yorkie (Yki), the *Drosophila* ortholog of the mammalian transcriptional coactivator yes-associated protein (YAP), as a missing link between Wts and transcriptional regulation. Yki is required for normal tissue growth and *diap1* transcription and is phosphorylated and inactivated by Wts. Overexpression of *yki* phenocopies loss-of-function mutations of *hpo* or *wts*, including elevated transcription of *cycE* and *diap1*, increased proliferation, defective apoptosis, and tissue overgrowth. Thus, Yki is a critical target of the Wts/Lats protein kinase and a potential oncogene.

Introduction

The increase in cell number that accompanies the growth of an organ or organism results from the balanced coordination of three simultaneous processes, including cell growth, cell proliferation, and cell death (reviewed by Conlon and Raff, 1999; Hipfner and Cohen, 2004). Cell growth is a prerequisite for cell proliferation during normal organ growth, and sustained cell proliferation must be coupled to appropriate cell growth. With appropriate cell growth, a net increase in cell number in a growing organ depends on the rate at which they are generated via cell proliferation, as well as the rate at which they are eliminated by cell death (apoptosis). How cell proliferation and cell death are coordinated during tissue growth and homeostasis is yet to be completely understood, and this mechanism must be intact throughout life to prevent diseases such as cancer.

Recent studies in mice and fruit flies have revealed two distinct modes in which cell proliferation and cell death could be coupled. In the first mode, increased

proliferation, such as that resulting from activation of the Myc oncogene, is coupled in an obligatory fashion to increased cell death. Such coupling between proliferation and apoptosis provides an important failsafe mechanism to prevent inappropriate proliferation of somatic cells (Lowe et al., 2004). In the second mode, increased proliferation, such as that resulting from activation of the microRNA *bantam*, or inactivation of the tumor suppressors *hippo* (*hpo*), *salvador* (*sav*), and *warts* (*wts*), is accompanied by an inhibition of cell death (reviewed by Hipfner and Cohen, 2004; Hay and Guo, 2003; Ryoo and Steller, 2003). Here, suppression of cell death might allow the overproliferating cells to overcome proliferation-induced apoptosis, thus resulting in a robust increase in organ size. In many aspects, these circumstances resemble certain cancer cells, which display both increased cell proliferation and suppressed cell death (Lowe et al., 2004).

hpo, *sav*, and *wts* (also called *lats*) were identified from genetic screens in *Drosophila* for negative regulators of tissue growth (Xu et al., 1995; Justice et al., 1995; Tapon et al., 2002; Kango-Singh et al., 2002; Harvey et al., 2003; Wu et al., 2003; Jia et al., 2003; Udan et al., 2003; Pantalacci et al., 2003). Inactivation of any of these genes results in increased cell proliferation and reduced apoptosis. *hpo* encodes a Ste20 family protein kinase, *sav* encodes a protein containing WW and coiled-coil domains, and *wts* encodes an NDR (nuclear Dbf-2-related) family protein kinase. Studies from several groups, including our own, have suggested that these genes function in a common pathway that coordinately regulates cell proliferation and apoptosis by targeting the cell-cycle regulator CycE and the cell-death inhibitor DIAP1 (Tapon et al., 2002; Kango-Singh et al., 2002; Harvey et al., 2003; Wu et al., 2003; Jia et al., 2003; Udan et al., 2003; Pantalacci et al., 2003). Using a combination of genetic and biochemical assays, we previously showed that Hpo, Sav, and Wts define a novel protein kinase cascade wherein Hpo, facilitated by Sav, phosphorylates Wts (Wu et al., 2003). We further demonstrated that this pathway, hereafter referred to as the Hpo pathway, negatively regulates the transcription of *diap1* (Wu et al., 2003). It is worth noting that our model differs significantly from an alternative model by others that suggests that this pathway regulates DIAP1 posttranscriptionally through phosphorylation of DIAP1 by Hpo (Tapon et al., 2002; Harvey et al., 2003; Pantalacci et al., 2003). Another unresolved issue in Hpo signaling concerns the molecular mechanism of the Wts/Lats kinase. While previous studies have identified a number of putative targets for this tumor suppressor, including the G2/M regulator cdc2 (Tao et al., 1999) and the actin regulators zyxin (Hirota et al., 2000) and LIMK1 (Yang et al., 2004), none of them could account for the excessive overgrowth associated with *wts* mutant clones. Thus, the most critical target of the Wts/Lats kinase has remained elusive.

Here we identify *yorkie* (*yki*) as the elusive target of the Wts/Lats tumor suppressor. *yki* encodes the *Drosophila* ortholog of yes-associated protein (YAP), a

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transcriptional coactivator in mammalian cells (Yagi et al., 1999; Strano et al., 2001; Vassilev et al., 2001). Yki is required for normal tissue growth and *diap1* transcription and is phosphorylated and inactivated by Wts. Overexpression of *yki* phenocopies loss-of-function mutations of *hpo*, *sav,* or *wts*. Taken together, our studies identify a missing link between Hpo signaling and transcriptional control and provide further support for our model implicating the Hpo signaling pathway in transcriptional regulation of *diap1*. Our studies further reveal a functional conservation between YAP and Yki and implicate YAP as a potential oncogene in mammals.

Results

Identification of Yki as a Wts Binding Protein

Our previous studies of the Hpo signaling pathway placed Wts as the most downstream component among Hpo, Sav and Wts. In an effort to extend this pathway further downstream, we carried out a yeast two-hybrid screen for Wts binding proteins. Using the noncatalytic N-terminal portion of Wts (1–608) as bait and from 1 million cDNA clones, we isolated three independent clones representing partial sequences of a gene annotated as CG4005 by the Berkeley *Drosophila* Genome Project (Figure 1A). We named this gene *yorkie* (*yki*) after Yorkshire Terriers, one of the world's smallest breeds of pet dogs, according to its loss-of-function phenotype (see later in Figure 4C). Consistent with the yeast two-hybrid results, Wts and Yki coimmunoprecipitate with each other in *Drosophila* S2 cells (Figure 1B).

Yki is most closely related to the human yes-associated protein (YAP, also called YAP65) (Sudol, 1994), with 31% identity between the two proteins (Figure 1C). Both proteins contain two WW domains, protein-protein interaction modules of 35–40 amino acids that are known to interact with PPXY-containing polypeptides (Macias et al., 2002). The similarity between Yki and YAP extends beyond the WW domains and includes a stretch of sequence similarity at the N-terminal part of the proteins (Figure 1C). While initially isolated as a protein that interacts with the SH3 domain of the Yes proto-oncogene, the involvement of YAP in Yes signaling has not been validated (Sudol, 1994). Notably, the corresponding SH3 binding region (Sudol, 1994) is absent in the *Drosophila* Yki protein (Figure 1C). On the other hand, YAP has been implicated as a transcriptional coactivator, a class of transcriptional regulators that do not bind to DNA themselves but associate with DNA binding transcription factors and supply or stimulate transcriptional activation of the cognate transcription factors. Specifically, YAP has been shown to function as a coactivator for a number of transcription factors, such as the p53 family transcription factor p73 (Strano et al., 2001), the Runt family protein PEBP2α (Yagi et al., 1999), and the TEAD/TEF family transcription factors (Vassilev et al., 2001). However, these studies have been performed exclusively in cultured mammalian cells and little is known about the physiological function of YAP.

The three independent Wts-interacting clones isolated from the yeast two-hybrid screen define the C-terminal half of Yki (residues 229–418) as a Wts binding region (Figure 1C). This region contains the two predicted WW domains, suggesting that the WW domains are required for Yki-Wts binding. Consistent with this hypothesis, mutating two critical residues of the WW domains abolished the binding between Yki and Wts (Figure 1B). Likewise, the N-terminal half of the Yki protein, which does not contain the WW domains, did not bind to Wts in the same assay (Figure 1B). Thus, the WW domains of Yki are required for its interaction with Wts.

Activation of *yki* Leads to Massive Tissue Overgrowth that Resembles the Loss-of-Function Phenotype of *hpo*, *sav*, or *wts*

To probe the physiological function of *yki*, we used the "flip-out" technique to generate clones of cells in which *yki* is overexpressed during development (Pignoni and Zipursky, 1997). *yki*-overexpressing clones led to marked overgrowth in adult epithelial structures (Figure 2A). Wing imaginal discs containing multiple *yki*-overexpressing clones could reach up to eight times the area of control wing discs raised under identical conditions (Figure 2C). Besides the overgrowth phenotype, adult cuticles secreted by *yki*-overexpressing cells display an unusual texture. In *yki*-overexpressing clones on the notum, the apical surface of the epidermal cells is domed such that cell-cell boundaries are visible between adjacent cells, whereas cell boundaries are not visible in the neighboring wild-type tissues (Figure 2B). Both the overgrowth and the abnormal cell morphology caused by *yki* overexpression closely resemble those shown previously for *hpo* and *wts* mutant cells (Wu et al., 2003; Justice et al., 1995), suggesting that these genes might function in a common pathway.

We measured cell-doubling time for control and *yki*-overexpressing cells in the wing imaginal disc by analyzing well-separated flip-out clones 48 hr post clone induction (Figures 2D and 2E). The cell-doubling time for wild-type and *yki*-overexpressing clones (30 pairs of clones analyzed) was 16.1 hr and 12.0 hr, respectively. Thus, like mutant clones of *hpo* or *wts*, *yki*-overexpressing cells multiply faster. Notably, while cells in the control clones intermingle with their neighbors and form wiggly borders, *yki*-overexpressing cells minimize their contacts with their neighbors and form round smooth borders (Figures 2D and 2E). This phenotype indicates distinct adhesive properties of the *yki*-overexpressing cells and resembles that seen with loss-of-function *wts* clones (Figures 2F and 2G). FACS analysis showed that *yki*-overexpressing cells have a similar cell-cycle profile and cell size distribution as compared to wild-type cells (Figure 2H). Thus, like loss-of-function of *hpo* (Wu et al., 2003), activation of *yki* does not accelerate a particular phase of the cell cycle. Rather, each phase of the cell cycle is proportionally accelerated.

Activation of *yki* in the Eye Imaginal Disc Leads to Increased Number of Interommatidial Cells without Affecting Photoreceptor Differentiation

For the rest of the study, we focused on the eye imaginal disc, a pseudostratified epithelium in which cell differentiation, proliferation, and apoptosis occur in a highly stereotyped manner (Wolff and Ready, 1993). In the third instar, the morphogenetic furrow (MF) tra-
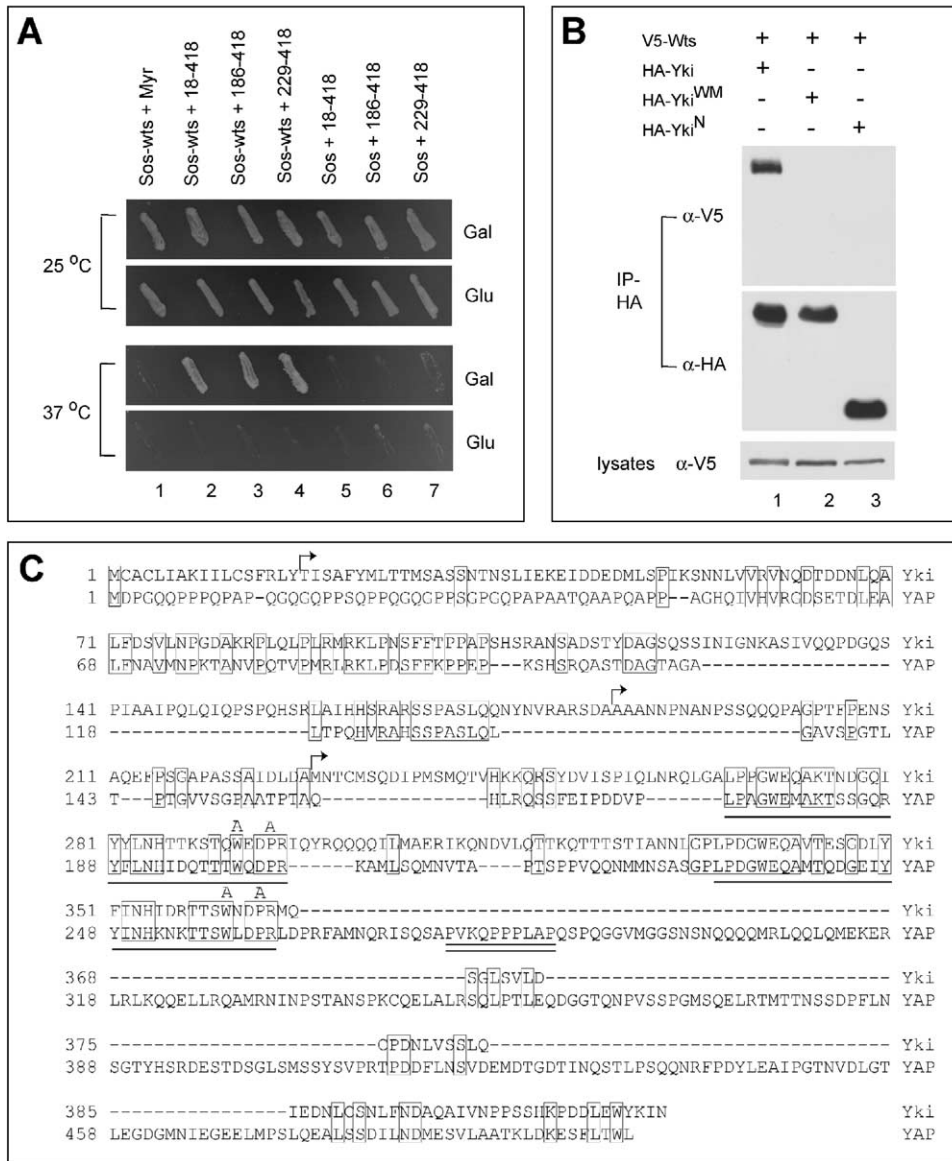
Figure 1. Identification of *yki*

(A) Isolation of Yki as a Wts binding protein by yeast two-hybrid. The *cdc25H* strain was transformed with the indicated plasmids and plated at permissive (25°C) and restrictive (37°C) temperature in galactose (prey expression induced) or glucose (prey expression suppressed) medium. Sos-Wts is the bait for library screening. Sos is empty bait containing Sos only. Myr is empty prey containing the myristylation signal only. 18–418, 186–418, 229–418 are three independent Yki clones isolated from the screen with the numbers representing the starting and ending positions of the Yki polypeptides in the clones.

(B) Physical association between Yki and Wts. HA-tagged wild-type Yki (Yki, lane 1), a mutant Yki carrying point mutations in the WW domains (Yki$^{W292A\ P295A\ W361A\ P364A}$, abbreviated as Yki^{WM} in lane 2), or a truncated Yki containing only the N-terminal half of the protein (Yki^N, lane 3) were coexpressed with V5-tagged Wts. α-HA immunoprecipitates were probed with α-V5.

(C) Alignment of Yki (418 aa) with the human YAP protein (504 aa). The starting position of the three Yki clones identified in the yeast two-hybrid screen is indicated by arrows. WW domains are indicated by single underlines. The Pro-rich motif of YAP that binds to the SH3 domain of Yes is indicated by double underlines. The alanine mutations that were used to mutate the WW domains in Yki^{WM} are also indicated.

verses the eye disc from posterior to anterior. Cells anterior to the MF are undifferentiated and divide asynchronously, whereas cells in the MF are synchronized in the G1 phase of the cell cycle. Posterior to the MF, cells either exit the cell cycle and differentiate or undergo one round of synchronous division (second mitotic wave, SMW) before differentiation. These cells assemble into approximately 750 ommatidia, leaving behind approximately 2000 superfluous cells that are eliminated by a wave of apoptosis ∼36 hr after puparium formation (APF).

To investigate whether activation of *yki* perturbs photoreceptor differentiation, we examined the neuronal marker Elav. As seen in Figures 2I–2I″, *yki*-overexpress-
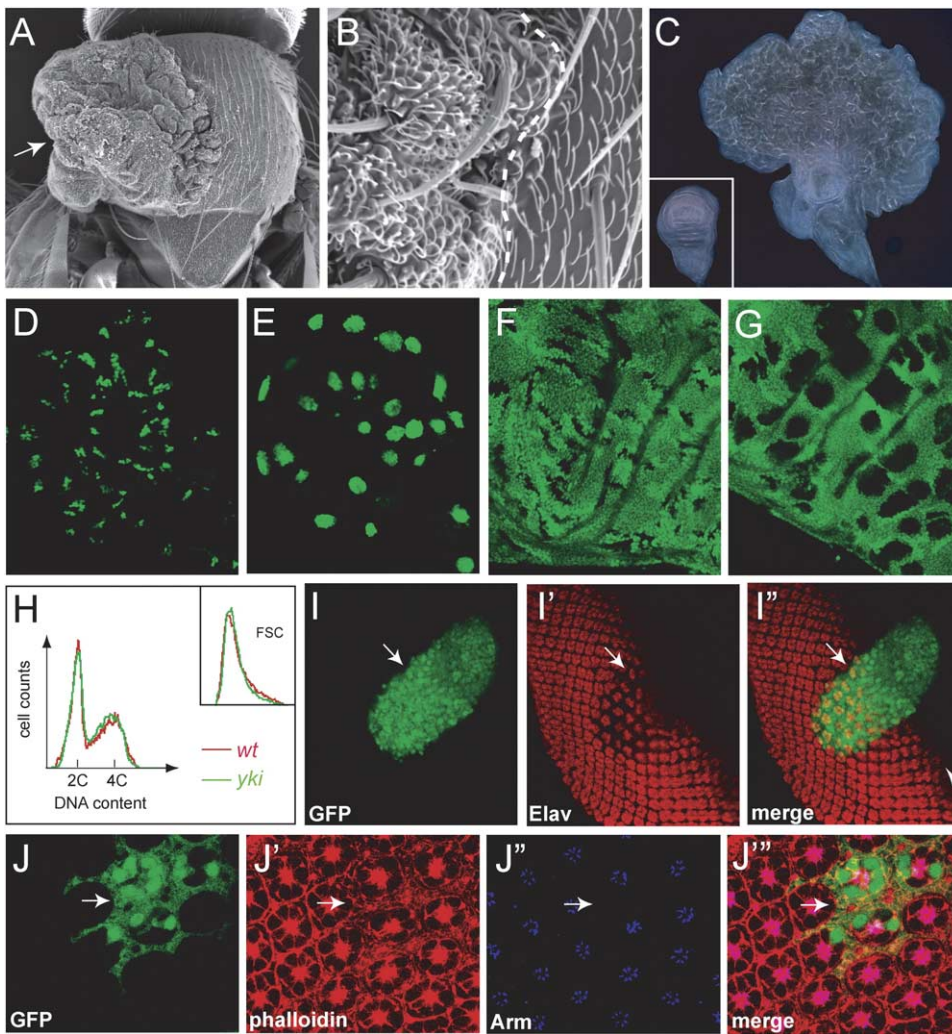
Figure 2. Overexpression of *yki* Drives Overgrowth

(A) Scanning electron micrograph of *Drosophila* notum showing a large overgrowth (arrow) caused by a *yki*-overexpressing clone.

(B) A high-magnification view of epidermal cells near the border of a *yki*-overexpressing clone on the notum. The dashed line marks the border between the wild-type cells and the mutant clone. The mutant clone is located to the left of the border. Note the honeycomb-like appearance of the *yki*-overexpressing cells, which was not seen in the neighboring wild-type epidermal cells.

(C) *yki*-overexpressing clones result in massive overgrowth of third instar wing discs. The inset at the lower left corner shows a sibling control wing disc without *yki* overexpression.

(D and E) Wing imaginal discs containing 48-hr-old control (D) and *yki*-overexpressing clones (E) generated by flip-out and positively marked by GFP. Note the difference in the sizes and shapes of the clones.

(F and G) Wing imaginal discs containing 48-hr-old control and *wts* mutant clones generated by FLP/FRT and marked by the absence of GFP. Note the difference in the sizes and shapes of the clones.

(H) Flow cytometric analysis. The DNA profiles of control and *yki*-overexpressing cells are indicated by red and green traces, respectively. The inset shows forward scattering (FSC), which measures cell size.

(I–I″) Third instar eye disc containing a *yki*-overexpressing clone (marked positively by GFP) and stained for the neuronal-specific Elav protein (red). Single-channel and composite images are shown as indicated. Note the presence of Elav-positive photoreceptor clusters in *yki*-overexpressing clone (indicated by arrows), as well as increased spacing between photoreceptor clusters in the clone. Arrowhead marks the MF.

(J–J″) Forty hour pupal eye containing a *yki*-overexpressing clone (marked positively by GFP) and stained for phalloidin (red), which highlights the outlines of the cells, and Armadillo (blue), which at this focal plane labels the apical cell surface of photoreceptors. Note the presence of a normal complement of photoreceptors and supernumerary interommatidial cells in the *yki*-overexpressing clone (indicated by arrows), as well as the increased spacing between photoreceptor clusters.

ing ommatidial clusters have the normal complement of photoreceptor cells. The spacing between adjacent ommatidial clusters is increased due to the presence of extra interommatidial cells (Figures 2I–2I″). The for-mation of extra interommatidial cells is most evident in pupal eye discs, when *yki*-overexpressing clones contain many additional cells between photoreceptor clusters (Figures 2J–2J″). Thus, like loss-of-function of *hpo*,
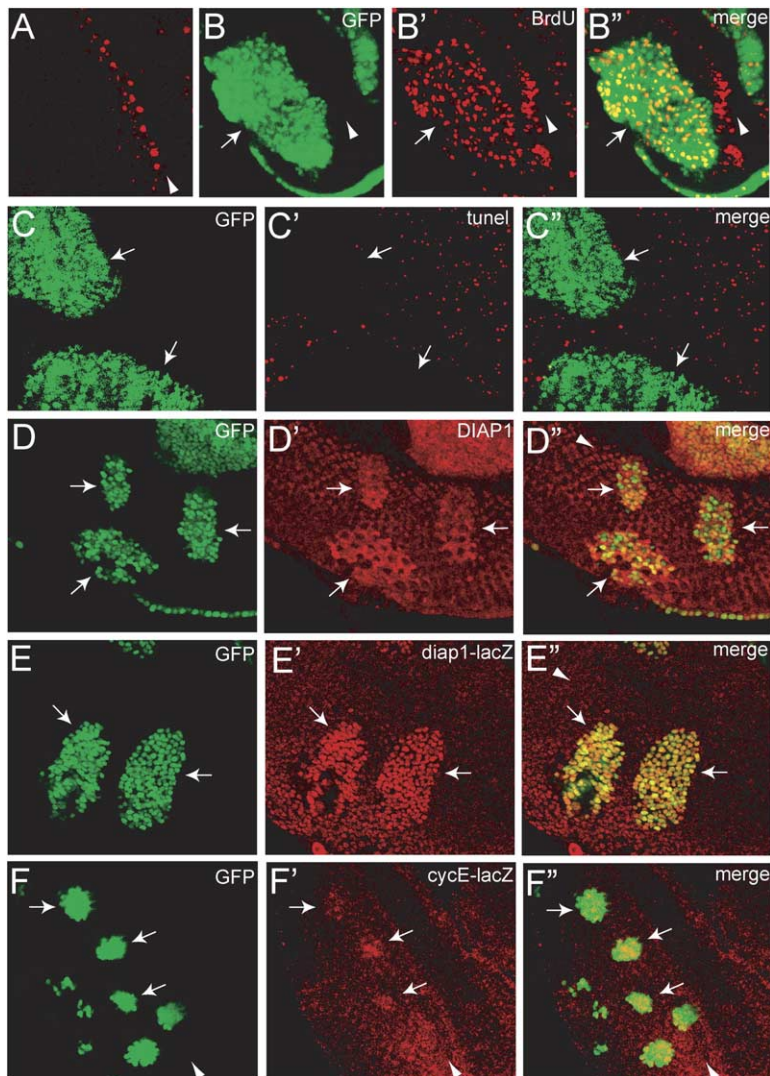
Figure 3. Overexpression of *yki* Promotes Cell Proliferation and Inhibits Apoptosis

In all panels, *yki*-overexpression clones were generated using the Act>CD2>Gal4 flip-out in conjunction with UAS-GFP and UAS-*yki* transgene and were positively marked by GFP (arrows). Single-channel and composite images are shown as indicated.

(A) Wild-type third instar eye disc labeled by BrdU incorporation (red). Note the synchronized band of S phase cells in the SMW (arrowhead).

(B–B″) Third instar eye disc containing *yki*-overexpressing clones and labeled with BrdU. Note the continued BrdU incorporation posterior to SMW in *yki*-overexpressing clones. Arrowhead marks the SMW.

(C–C″) Thirty-six hour APF pupal eye containing *yki*-overexpressing clones and labeled with TUNEL. Cell death is absent in *yki*-overexpressing clones but abundant in the neighboring wild-type cells.

(D–D″) Third instar eye disc containing *yki*-overexpressing clones and stained with α-DIAP1 antibody. Note the cell-autonomous increase in DIAP1 protein levels in *yki*-overexpressing cells.

(E–E″) *yki*-overexpressing clones were generated in flies containing the *diap1-lacZ* reporter *th^{j5c8}* and stained for lacZ protein. Note the cell-autonomous increase in *diap1-lacZ* expression in *yki*-overexpressing cells.

(F–F″) *yki*-overexpressing clones were generated in flies containing a *cycE-lacZ* reporter and stained for lacZ protein. Arrowhead marks the MF. Note a modest increase in *cycE-lacZ* expression in *yki*-overexpressing cells.

sav, or *wts*, *yki* overexpression results in an increased number of uncommitted, interommatidial cells without affecting early retina patterning.

Activation of *yki* Leads to Increased Cell Proliferation and Decreased Apoptosis

To pinpoint the developmental cause of *yki*-induced overgrowth, we monitored cell proliferation and apoptosis in eye imaginal discs. In wild-type eye discs (Figures 3A), cells posterior to the MF undergo a synchronous second mitotic wave (SMW) that can be revealed as a band of BrdU-positive cells. Few BrdU-positive cells are found posterior to the SMW. In *yki*-overexpressing clones, cells fail to undergo cell-cycle arrest posterior to the SMW and continue cell cycles as shown by BrdU incorporation (Figures 3B–3B″) as well as M phase marker phospho-histone H3 (PH3) (data not shown). Thus, *yki* overexpression results in increased cell proliferation.

Using the TUNEL assay, we monitored cell death in the pupal retina when a wave of apoptosis normally re-

moves excess interommatidial cells around 36 hr APF. Strikingly, cell death was significantly suppressed in *yki*-overexpressing clones, even though abundant apoptosis was detected in the neighboring wild-type cells (Figures 3C–3C″). Thus, normal developmental cell death is largely inhibited by *yki* overexpression.

Activation of *yki* Leads to Increased Transcription of *diap1* and *CycE*

The increased cell proliferation and decreased apoptosis resulting from *yki* overexpression are strikingly similar to those caused by loss of *hpo*, *sav*, or *wts*, suggesting that Yki functions in the Hpo pathway. To further explore this possibility, we examined the transcription of cell-death inhibitor *diap1* and cell-cycle regulator *cycE*, known targets of the Hpo pathway (Wu et al., 2003). Elevated DIAP1 protein was detected in *yki*-overexpressing clones in the eye discs (Figures 3D–3D″). This regulation is largely mediated at the level of *diap1* transcription since the expression of *th^{j5c8}*, a P[lacZ] enhancer trap reporter inserted into the *diap1*

locus, was similarly elevated in *yki*-overexpressing clones in a cell-autonomous manner (Figures 3E–3E″). A *cycE-lacZ* reporter containing 16.4 kb of the 5′ regulatory sequence of *cycE* (Jones et al., 2000) was also increased in *yki*-overexpressing clones, especially those close to the MF (Figures 3F–3F″), although the effect was less profound than that observed with the *diap1* reporter. Thus, like loss of *hpo*, *sav*, or *wts*, overexpression of *yki* results in increased transcription of *diap1* and *cycE*. It is worth noting that our previous analyses of *hpo* mutant clones also revealed a "tighter" regulation of *diap1*: while *diap1* transcription is elevated in all *hpo* mutant cells irrespective of their relative position to the MF, *cycE* transcription is only elevated in *hpo* mutant cells close to the MF (Wu et al., 2003). These observations suggest that *diap1* might represent a more direct transcriptional target of the Hpo pathway.

yki Is Required for Tissue Growth and Normal *diap1* Transcription

To further explore the role of Yki in Hpo signaling, we generated a loss-of-function mutation of *yki* by homologous recombination (Gong and Golic, 2003). Our targeting construct was designed in such a way that all of the coding sequence of *yki* was replaced by the *w*+ marker, thus resulting in a null allele (Figure 4A). *yki* null mutants are homozygous lethal and die as late embryos and early first instar larvae. A full-length *yki* cDNA driven by the ubiquitous α-tubulin promoter completely rescued *yki* null animals to viable and phenotypically normal adult flies.

We used *eyeless*-FLP to selectively remove *yki* function in over 90% of the eye disc cells (Newsome et al., 2000). Eyes composed predominantly of *yki* mutant cells were markedly reduced in size when compared to control animals (Figures 4B–4B′ and 4C–4C′), thus revealing an essential function for *yki* in tissue growth. To follow *yki* mutant cells during development, we used FLP/FRT to examine genetically marked clones of *yki* mutant cells. *yki* mutant clones generated at 40 hr AED were hardly observed in third instar wing discs, with rare clones recovered containing only a few cells (Figures 4D–4D″). *yki* mutant clones generated at a similar stage were more frequently recovered in the eye discs but contained much fewer cells than the wild-type twin spots (Figure 4F). Despite the severe growth defects, loss of *yki* did not perturb early retina differentiation, as shown by the normal expression of the neuronal marker Elav (Figure 4E). Taken together, these results reveal a specific requirement for *yki* in tissue growth.

To further probe the requirement of Yki in the Hpo pathway, we examined *diap1* transcription in *yki* mutant clones using the *th*[i5c8] *diap1-lacZ* reporter. Consistent with the overexpression results (Figure 3), *diap1-lacZ* expression was reduced in *yki* null cells in a cell-autonomous manner (Figure 4F). Similar results were seen in the wing discs (data not shown). DIAP1 protein level was also reduced in a cell-autonomous manner in *yki* mutant clones (see later, Figures 7A–7A″). Thus, *yki* is required for the normal level of *diap1* transcription in *Drosophila*.

The Transcriptional Coactivator Activity of Yki Is Negatively Regulated by the Hpo Pathway

The results presented so far are consistent with a model wherein Yki acts antagonistically to Hpo, Sav, and Wts in a common signaling pathway that coordinately controls cell proliferation and apoptosis. Based on the physical interactions between Yki and Wts (Figure 1), and given that YAP, the mammalian homolog of Yki, is known to function as a transcriptional coactivator (Yagi et al., 1999; Strano et al., 2001; Vassilev et al., 2001), we further hypothesized that Yki functions downstream of Wts to regulate transcription of genes such as *diap1* and that the Hpo pathway negatively regulates the coactivator activity of Yki.

To test our hypothesis that the coactivator activity of Yki is negatively regulated by the Hpo pathway, we first established a transcription assay for Yki activity in *Drosophila* S2 cells. Since the cognate transcription factor(s) that partner with Yki are not yet identified, we fused Yki to the DNA binding domain (DB) of the yeast Gal4 transcription factor (Figure 5A). The activity of this fusion construct was then assayed using a Gal4-responsive reporter. Consistent with previous reports of YAP as a transcriptional coactivator in mammalian cells, the Gal4DB-Yki fusion protein exhibited potent transcriptional activation (Figure 5B). Strikingly, transcriptional activity of the Gal4DB-Yki fusion was abolished when Hpo, Sav, and Wts plasmids were coexpressed (Figure 5B). This effect is specific to Yki since activity of the full-length Gal4 (with its own activation domain) was unaffected by the coexpression of Hpo, Sav, and Wts (Figure 5B). These results suggest that the Hpo pathway negatively regulates the coactivator activity of Yki.

Dosage-Sensitive Genetic Interactions between *yki* and the Hpo Pathway

To further probe a functional link between *yki* and the Hpo pathway, we investigated their genetic interactions. We have shown previously that while expression of *hpo* or *wts* directly from the GMR promoter results in viable flies with rough or slightly rough eyes, respectively, cointroduction of GMR-*hpo* and GMR-*wts* into the same animals results in 100% lethality at early pupal stage (Wu et al., 2003). Strikingly, such lethality was completely rescued by coexpression of *yki* from a GMR-*yki* transgene (Figure 5C). Interestingly, this lethality was also completely rescued by coexpression of the human *YAP* gene (Figure 5C). In another line of experiments, we took advantage of the complete pupal lethality caused by the overexpression of UAS-*hpo* driven by the GMR-Gal4 driver (Figure 5D). Interestingly, this lethality was also rescued by the coexpression of *yki* (100% rescue) or *YAP* (21% rescue) (Figure 5D). Taken together, these genetic interactions further support our model that Yki acts antagonistically to Hpo, Sav, and Wts in a common signaling pathway. The ability of a human YAP transgene to rescue the lethality of flies caused by Hpo pathway hyperactivation reveals a functional conservation between Yki and YAP, suggesting that YAP might play a similar role in mammalian growth control.
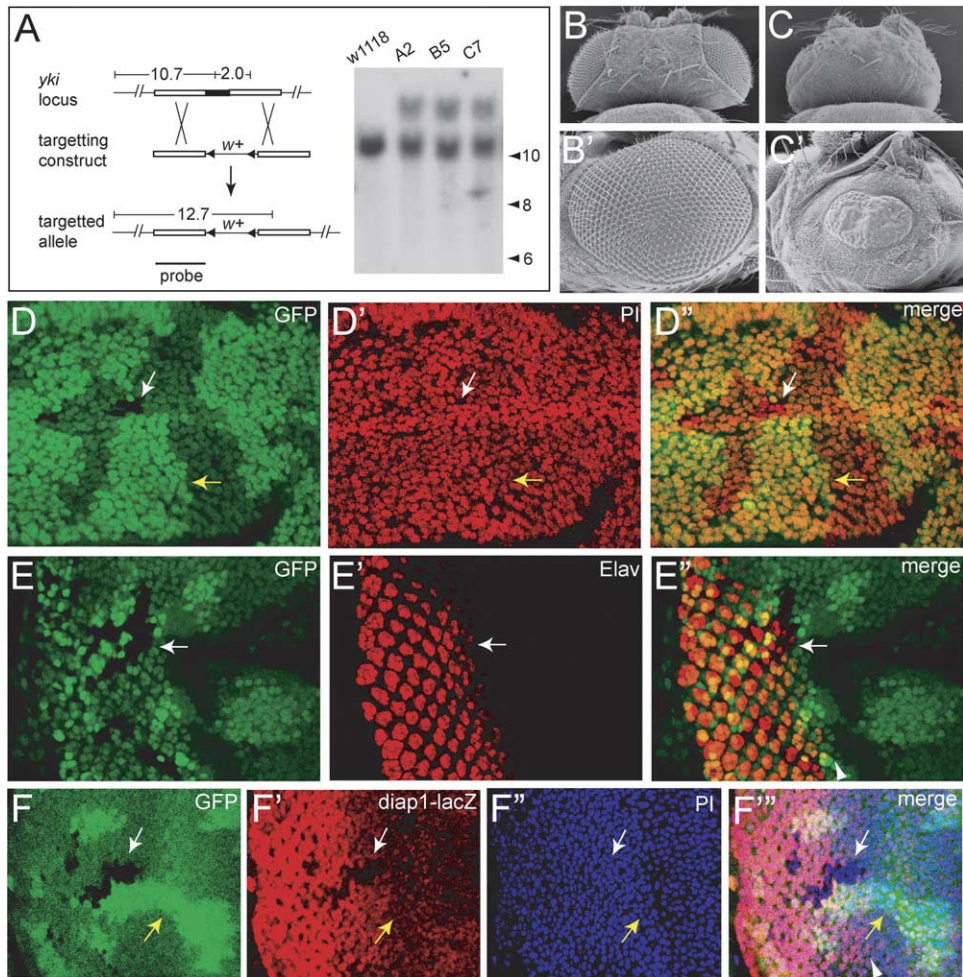
Figure 4. *yki* Is Required for Tissue Growth and Transcriptional Regulation of *diap1*

For confocal images, single-channel and composite pictures are shown as indicated.

(A) Generation of *yki* knockout by gene targeting. The diagram at left shows organization of the endogenous *yki* locus, the targeting construct, and the expected targeted allele. DNA probe used for Southern blotting is shown as a solid line, and the sizes (in kilobases) of expected BamHI fragments are indicated. The right shows Southern blotting of BamHI-digested genomic DNA hybridized with the indicated probe. DNA was extracted from wild-type flies (w^{1118}) and three independent *yki* knockout lines recovered from gene targeting (as heterozygotes over the CyO balancer). The size of the molecular weight marker is indicated to the right of the autoradiograph. While the endogenous *yki* locus produces a 10.7 kb fragment, the targeted allele produces a 12.7 kb band.

(B and B′) Scanning electron micrographs (SEM) of wild-type flies showing a dorsal view of the head (B) and a side view of the compound eye (B′). The genotype is *y w ey-flp; FRT42D/FRT42D w⁺ l(2)c1-R11.*

(C and C′) SEM of fly heads composed predominantly of cells lacking *yki* function. A dorsal view (C) and a side view (C′) are shown. The genotype is *y w ey-flp; FRT42D yki^B5/FRT42D w⁺ l(2)c1-R11.*

(D–D″) Third instar wing disc containing *yki* mutant clones (induced by FLP/FRT at 40 hr AED) and stained with propidium iodide (PI). Homozygous –/– clones, marked by the absence of GFP, were largely undetectable, with rare exceptions containing 1–3 cells (white arrow). In contrast, the sibling +/+ twin spots, marked by the 2XGFP signal (darker green, yellow arrow), were readily observed.

(E–E″) Third instar eye disc containing *yki* mutant clones (marked by lack of GFP) and stained for the neuronal-specific Elav protein. Mutant clones were induced at 60 hr AED to allow for more efficient recovery of *yki* mutant cells. Note the presence of Elav-positive cells in *yki* mutant clones (arrow). Arrowhead marks the MF.

(F–F″) *yki* mutant clones (marked by lack of GFP) were generated in flies containing the *diap1-lacZ* reporter, and a third instar eye disc was stained for lacZ (red) and the nuclear stain PI (blue). Note the cell-autonomous decrease in *diap1-lacZ* levels in *yki* mutant clones (white arrow) and the smaller size of the *yki* mutant clone as compared to the twin spots (yellow arrow). Arrowhead marks the MF.

Phosphorylation of Yki by Wts

Given the direct interaction between Yki and Wts (Figure 1) and that Wts encodes a protein kinase, we hypothesized that Yki is regulated by the Hpo pathway through Wts-mediated phosphorylation. To test this possibility, we first examined phosphorylation of Yki by the Hpo pathway using an S2 cell-based assay. As shown in Figure 6A, coexpression of Wts and Yki resulted in a small mobility retardation of Yki. Coexpression of Hpo-Sav with Yki also resulted in a mobility shift of Yki, and coexpression of Hpo-Sav-Wts resulted in an even greater mobility shift of Yki. The mobility shift of
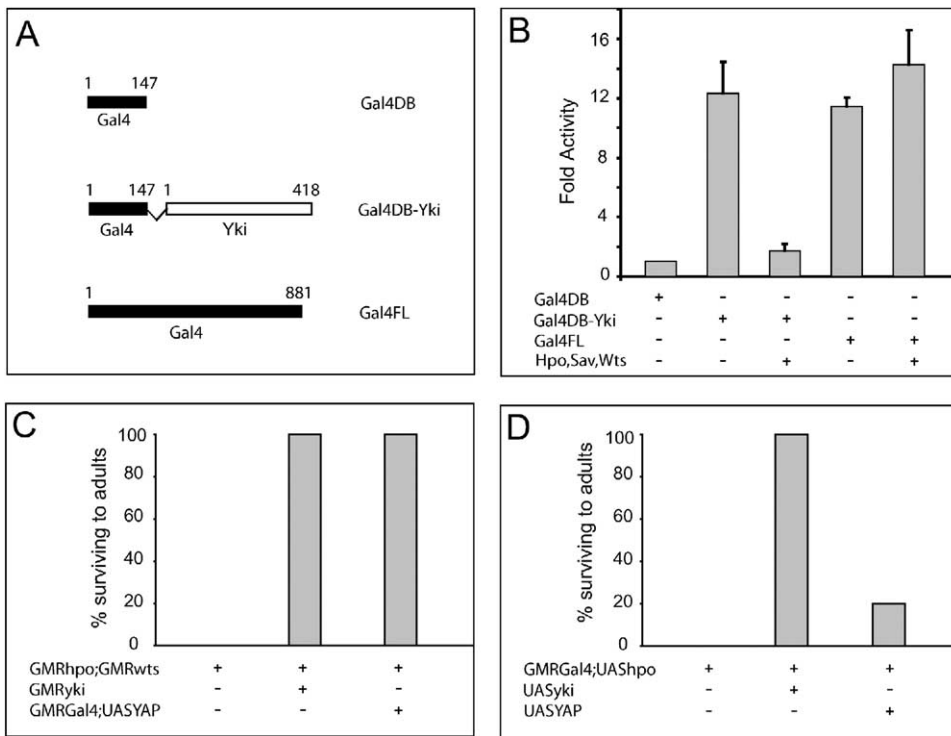
Figure 5. The Hpo Signaling Pathway Antagonizes the Transcriptional Coactivator Activity of Yki

(A) Schematic diagram of Gal4-related constructs used in the S2 cell transcription assay.

(B) Coactivator activity of Yki and its negative regulation by the Hpo pathway. S2 cells were transfected with the indicated plasmids along with a Gal4-responsive luciferase reporter. Luciferase activities relative to those obtained with Gal4 DNA binding domain alone (Gal4DB) are plotted. Error bars represent standard deviation from three independent transfections.

(C) Antagonistic, dosage-sensitive interactions between *yki* and *hpo-wts*. The percentage of flies surviving to adults is shown for the indicated genotypes (>200 animals scored for each genotype). Note the complete lethality of GMR-*hpo*; GMR-*wts* animals, which was completely reversed by GMR-*yki*, or expression of the human *YAP* gene under the GMR-Gal4 driver (GMR-Gal4; UAS-*YAP*).

(D) Genetic interactions between *yki* and *hpo*. The percentage of flies surviving to adults is shown for the indicated genotypes (>200 animals scored for each genotype). The lethality of GMR-GAL4; UAS-*hpo* animals was completely and partially rescued by *yki* and *YAP*, respectively (crosses done at 18°C).

Yki induced by Hpo-Sav-Wts expression was abrogated by phosphatase treatment, demonstrating that this shift is due to protein phosphorylation (Figure 6B). It is worth noting that the increasing phosphosphorylation of Yki induced by Wts, Hpo-Sav, and Hpo-Sav-Wts in the S2 cell assay (Figure 6A) correlates with the severity of the overexpression phenotype caused by the respective transgenes in vivo: expression of Wts by the GMR promoter results in slightly rough eyes, expression of Hpo-Sav results in strong rough eyes with reduced size, and expression of Hpo-Sav-Wts results in complete animal lethality (Tapon et al., 2002; Wu et al., 2003). These results suggest that Yki phosphorylation is a relevant output of the Hpo signaling pathway.

To determine whether Yki is a direct substrate of Wts, we carried out in vitro kinase assays. When expressed alone, Wts had little kinase activity on Yki (Figure 6C, lane 1). When coexpressed with Hpo-Sav, however, Wts displayed specific kinase activity on Yki (Figure 6C, lane 2) but not a control substrate (Figure 6C, lane 4). Moreover, a kinase-dead mutation of Wts completely abolished the in vitro kinase activity of Wts toward Yki (Figure 6C, lane 3). These data confirm that Yki is a kinase substrate of Wts. Furthermore, the observation that Hpo-Sav coexpression stimulates the kinase activity of Wts on Yki is consistent with our previous report showing activation of Wts by Hpo-Sav as measured by the phosphorylation status of Wts (Wu et al., 2003).

If Hpo-Sav activates Wts, which in turn phosphorylates Yki, one would predict that the mobility shift of Yki induced by transfected Hpo-Sav or Wts in our S2 cell assay (Figure 6A) might require the endogenous Wts or Hpo, respectively. Indeed, RNAi of *wts* completely reversed the mobility shift of Yki induced by Hpo-Sav expression (Figure 6D), and RNAi of *hpo* completely reversed the mobility shift of Yki induced by Wts expression (Figure 6E). These data further support our model that Yki is phosphorylated by Wts upon activation of the Hpo pathway.

yki Is Genetically Epistatic to *hpo*, *sav*, and *wts*

The genetic evidence presented so far suggests that *yki* acts antagonistically to *hpo*, *sav*, and *wts*. Our biochemical studies further refined this model and demonstrate that Yki is phosphorylated and inactivated by the Hpo pathway via Wts-mediated phosphorylation. A pre-
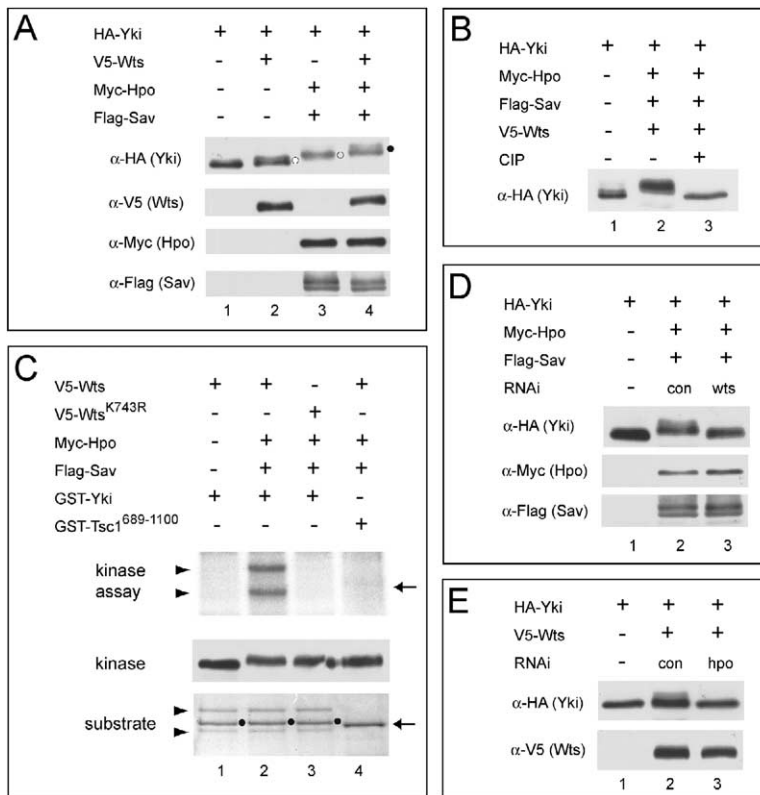
Figure 6. Phosphorylation of Yki by Wts

(A) Lysates from S2 cells expressing various epitope-tagged proteins were probed with indicated antibodies. Increasingly phosphorylated forms of Yki are indicated by small circles next to the protein bands and filled with white, gray, and black colors.

(B) Phosphatase (CIP) treatment reversed the mobility shift of Yki induced by Hpo-Sav-Wts.

(C) Wts phosphorylates Yki in vitro. V5-tagged Wts or kinase-dead Wts^{K743R} was expressed alone or together with Hpo-Sav in S2 cells, immunoprecipitated, and tested for kinase activity against GST-Yki and GST-Tsc1 (as a control substrate). The signal of Yki phosphorylation by Wts is indicated by arrowheads, and the arrow marks the expected migration position of GST-Tsc1 on autoradiograph (top gel). The input kinase and substrate are also shown (bottom two gels). Note that our GST-Yki preparation contains two Yki-related bands (arrowheads) as well as a contaminating band (black dots, see Experimental Procedures for details). Note that only the Yki-related bands are phosphorylated, and that Yki phosphorylation is only detected when Wts is coexpressed with Hpo-Sav (lane 2).

(D) S2 cells were transfected with the indicated plasmids along with control or wts dsRNA and probed with indicated antibodies. Note suppression of the Yki mobility shift by wts RNAi.

(E) S2 cells were transfected with the indicated plasmids along with control or hpo dsRNA and probed with indicated antibodies. Note suppression of the Yki mobility shift by hpo RNAi.

diction of this model is that loss-of-function mutations of *yki* should be genetically epistatic to those of *hpo*, *sav*, or *wts*. To test this hypothesis, we generated clones of cells that are doubly mutant for *hpo-yki*, *sav-yki*, or *wts-yki*. While loss of *hpo*, *sav*, or *wts* results in increased *diap1* transcription and overgrowth (Wu et al., 2003), *hpo-yki*, *sav-yki*, or *wts-yki* double mutant clones displayed phenotypes indistinguishable from those of *yki* mutant clones, including retarded growth, decreased DIAP1 protein levels, and decreased *diap1* transcription (Figure 7). These genetic observations further strengthen our molecular model implicating Yki as a target of Wts in the Hpo pathway.

Discussion

The mechanisms of how body and organ size are regulated are just beginning to be understood (Conlon and Raff, 1999; Hipfner and Cohen, 2004). Recent studies in *Drosophila* have implicated a number of pathways in the coordinate control of cell growth, proliferation, and apoptosis, which ultimately regulate body and organ size. The insulin/Tsc/TOR signaling network, for example, plays a major role in coordinating organ growth with environmental cues such as nutrients (Hafen, 2004; Pan et al., 2004). The Hpo signaling pathway, on the other hand, might contribute to an intrinsic size

"checkpoint" that normally stops growth when a given organ reaches its characteristic size. Thus, molecular elucidation of the Hpo signaling pathway should provide important insights into size-control mechanisms in development.

Identification of Yki as a Direct Target of Wts/Lats in the Hpo Pathway

Previous studies of the Wts/Lats tumor suppressor have failed to identify any target of this kinase that could account for its potent growth-regulatory activity. Here, we provide genetic and biochemical evidence implicating Yki, the *Drosophila* ortholog of the mammalian coactivator protein YAP, as a direct, critical target of Wts/Lats in the Hpo pathway (Figure 8). Yki associates with and is phosphorylated by Wts. Moreover, Wts-mediated phosphorylation of Yki is stimulated by upstream components of the Hpo pathway, and the extent of Yki phosphorylation induced by Hpo pathway components in vitro correlates with the severity of the overexpression phenotype caused by the respective transgenes in vivo. Most importantly, overexpression of *yki* phenocopies loss of *hpo*, *sav*, or *wts*, while loss of *yki* results in the opposite phenotype, and our epistasis analyses unambiguously placed *yki* downstream of *hpo*, *sav*, and *wts*. Taken together, these results provide compelling evidence that Yki is a critical target of Wts/
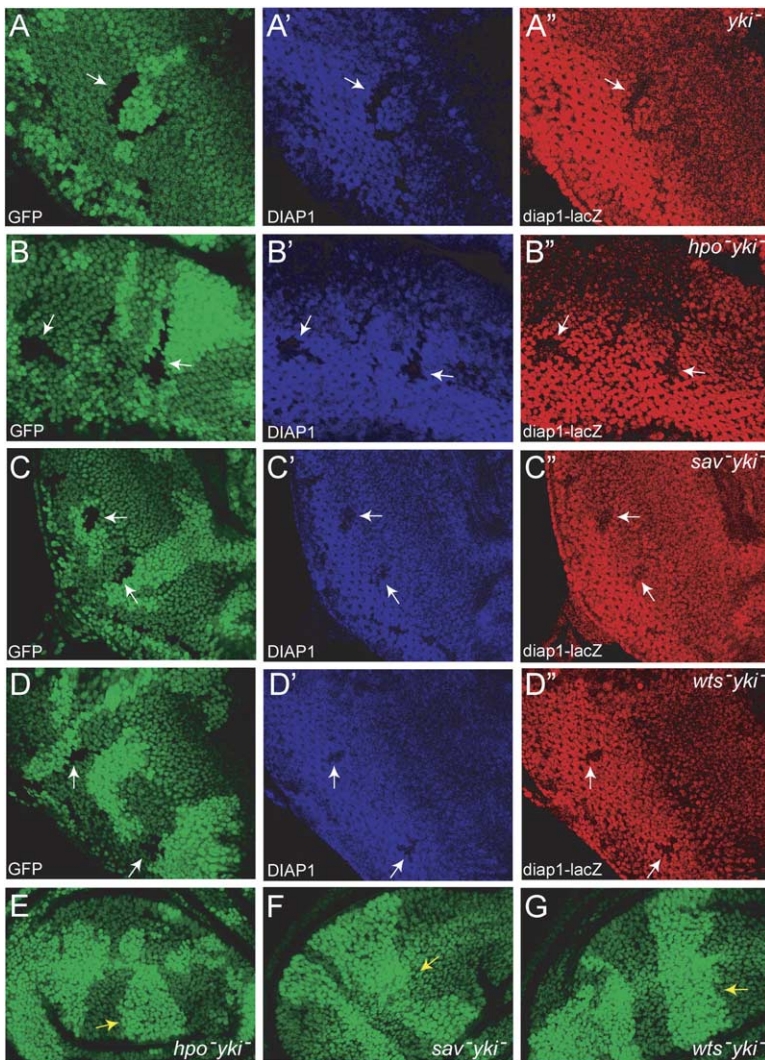
Figure 7. *yki* Is Genetically Epistatic to *hpo*, *sav*, and *wts*

In all panels, mutant clones were generated at 40 hr AED and marked by the lack of GFP. (A)–(D″) show eye imaginal discs, and (E)–(G) show wing imaginal discs. Single-channel and composite confocal images are shown as indicated.

(A–D″) Third instar eye disc containing *yki* (A–A″), *hpo-yki* (B–B″), *sav-yki* (C–C″), and *wts-yki* (D–D″) mutant clones in the presence of *th^{j5c8}* and stained with antibodies against DIAP1 (blue) and lacZ (red). Note that all the mutant clones show a cell-autonomous decrease of DIAP1 and *diap1-lacZ*, as well as growth disadvantage as compared to the twin spots (marked by 2XGFP signal, darker green).

(E–G) Third instar wing discs containing double mutant clones of *hpo-yki* (E), *sav-yki* (F), and *wts-yki* (G). Note the presence of +/+ twin spots, marked by 2XGFP signal (yellow arrow), and a general absence of homozygous mutant cells (marked by the absence of GFP). This phenotype is similar to *yki* wing clones generated at 40 hr AED (see Figures 4D–4D″).

Lats in the Hpo pathway. We further speculate that the relationship between Yki and Hpo signaling is likely conserved during evolution since overexpression of mammalian YAP was able to rescue the lethality associated with hyperactivation of the Hpo pathway in *Drosophila*. The functional conservation between Yki and YAP further suggests that YAP might function as an oncogene in mammals.

To our knowledge, Yki is the first substrate identified for NDR family kinases, which include, besides Wts/Lats, Cbk1, Dbf2, and Dbf20 in budding yeast, Sid2 and Orb6 in fission yeast, Cot-1 in Neurospora, Sax-1 in *C. elegans*, Trc in *Drosophila*, and NDR1 and NDR2 in mammals (reviewed by Tamaskovic et al., 2003). The NDR family kinases are involved in diverse events in cell-cycle and cell morphogenesis, such as maintaining cell polarity (Cbk1 and Orb6), coordinating CDK inactivation and cytokinesis (Dbf2, Dbf20, and Sid2), and neuronal morphogenesis (Sax-1). Despite their diverse cellular functions, all NDR family kinases share similar structural features, such as the insertion of 30–60 amino acids between kinase subdomains VII and VIII,

the presence of conserved activation loop and hydrophobic motif, and the presence of N-terminal noncatalytic domain (Tamaskovic et al., 2003). These common features suggest that NDR family kinases may employ similar mechanisms to interact with their substrates and regulators. Along this line, we suggest that the approach described in this study, which uses the N-terminal noncatalytic domain of Wts as yeast two-hybrid bait, might provide a general method to discover substrates for other NDR family kinases.

Regulation of NDR Family Kinases by Ste20-like Kinases

In our previous studies of the Hpo pathway, we proposed a model whereby Hpo, somehow facilitated by Sav, phosphorylates Wts (Wu et al., 2003). While our model implied that phosphorylation of Wts leads to activation of its kinase activity, we were previously unable to directly test this due to the lack of an appropriate assay that measures pathway activity downstream of Wts. The identification of Yki as a Wts substrate provides a new tool to evaluate our earlier model. Consis-
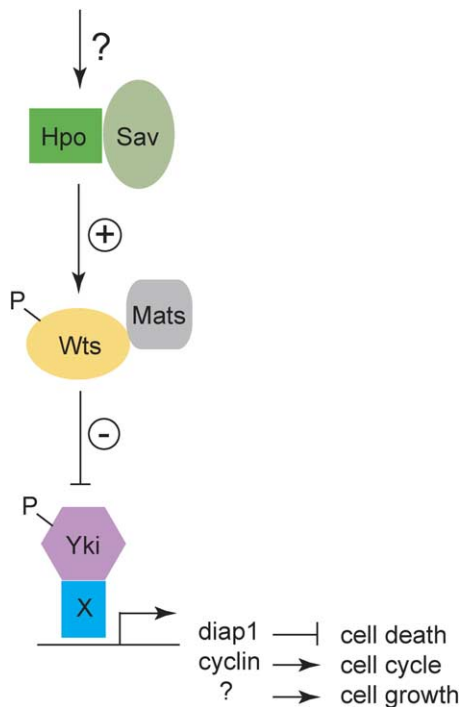
Figure 8. A Model of the Hpo Signaling Pathway in the Control of Organ Growth

An unknown signal controls Hpo, which in turn phosphorylates and activates Wts. The activation of Wts by Hpo is potentiated by Sav and Mats, which have been shown to associate with Hpo and Wts, respectively. The exact mechanism of this potentiation remains to be determined. The activated Wts kinase phosphorylates and inactivates the transcriptional coactivator Yki, which normally partners with an unknown DNA binding transcription factor (X) to activate gene transcription. Transcriptional targets of the Yki-X transcription complex include the cell-death inhibitor *diap1* and possibly cell-cycle regulators such as cyclin E. There also likely exist target(s) of the Yki-X complex that regulate cell growth since cell growth must be proportionally stimulated to sustain the increased proliferation of *hpo*, *sav*, *wts*, and *mats* mutant cells. It remains to be determined how the activity of the Hpo signaling pathway is regulated during the growth of imaginal discs.

tent with our previous model implicating Hpo as an activating kinase of Wts, we show that in S2 cells, the phosphorylation of Yki induced by transfected Wts is dependent on the endogenous Hpo protein (Figure 6E). Furthermore, the in vitro kinase activity of Wts toward Yki is strongly stimulated when Wts is coexpressed with Hpo-Sav. We suggest that such a relationship between Hpo and Wts is likely conserved during evolution. Indeed, a recent study has demonstrated the activation of the mammalian Lats1 kinase by the mammalian Hpo homologs Mst1/Mst2 (Chan et al., 2005).

It is worth noting that several Ste20-like kinases have been implicated in the activation of NDR kinases. Such examples include the activation of Wts by Hpo (Wu et al., 2003; Chan et al., 2005), the activation of Dbf2 by Cdc15 (Mah et al., 2001), the regulation of Orb6 by Pak1 (Verde et al., 1998), and the regulation of Sid2 by Sid1 (Guertin et al., 2000). Thus, activation by Ste20-like kinases might represent a general mechanism for regu-

lating NDR kinases. In retrospect, the difficulties in identifying substrates for NDR kinases might be due to their substrate specificity in conjunction with a requirement for activation by upstream kinases. Another emerging feature of the NDR kinases concerns their regulation by the Mob family of small regulatory proteins, which have been found to associate with multiple NDR family kinases, such as Dbf2, Orb6, Sid2, Cbk1, NDR1, and NDR2 (Tamaskovic et al., 2003). In *Drosophila*, Mats, a Mob family protein, has recently been identified as a tumor suppressor gene that likely regulates Wts in the Hpo signaling pathway (Lai et al., 2005). Thus, regulation by Mob family proteins likely represents an important and shared feature of modulating NDR family kinases.

Transcriptional Regulation of *diap1* by the Hpo Signaling Pathway

Previous studies of Hpo signaling suggested two contrasting models on how this pathway regulates the cell-death regulator DIAP1. Using a *diap1-lacZ* reporter to follow *diap1* transcription, we have observed elevated *diap1* transcription in mutant clones of *hpo*, *sav*, or *wts* that closely matches the increase in DIAP1 protein levels. Based on these results, we proposed that the Hpo pathway negatively regulates *diap1* at the level of transcription (Wu et al., 2003). However, an alternative model suggested that Hpo regulates DIAP1 posttranscriptionally by directly phosphorylating DIAP1, thus promoting its degradation (Tapon et al., 2002; Harvey et al., 2003; Pantalacci et al., 2003). This model was largely based on two lines of evidence, including in situ hybridization showing unchanged *diap1* mRNA level in mutant clones and the ability of Hpo to phosphorylate DIAP1 in vitro. We note, however, that in situ hybridization used in the latter studies did not involve the marking of mutant clones and thus may be less definitive than the *diap1-lacZ* reporter. A major drawback of the posttranscriptional model is that it cannot easily account for the involvement of Wts in the Hpo pathway. A direct link between Hpo and DIAP1 inevitably implies Wts as acting upstream or in parallel with Hpo, which is contradictory to other studies of the NDR kinases that generally place them downstream of the Ste20-like kinases (Wu et al., 2003; Chan et al., 2005; Mah et al., 2001; Verde et al., 1998; Guertin et al., 2000).

If the Hpo signaling pathway regulates *diap1* via a transcriptional mechanism, then there should exist transcriptional regulator(s) that control *diap1* transcription and whose activity may be regulated by the Hpo signaling pathway. Furthermore, such transcriptional regulator(s) must account for the mutant phenotypes resulting from deregulation of the Hpo pathway, such as changes in *diap1* transcription and overgrowth. Our current study demonstrates that Yki represents such a regulator, thus further supporting our previous model implicating the Hpo pathway in regulating *diap1* transcription.

Understanding the molecular mechanisms by which the Hpo pathway regulates *diap1* transcription will provide important insights into the developmental coordination of tissue growth and apoptosis. Like other coactivators, Yki presumably functions by interacting with

DNA binding transcription factors. YAP, the mammalian homolog of Yki, is known to function as coactivator for a number of transcription factors, such as the p53 family member p73 (Strano et al., 2001), the Runt family member PEBP2α (Yagi et al., 1999), and the four TEAD/TEF transcription factors (Vassilev et al., 2001). This interaction is generally mediated by WW domains of YAP and PPxY motifs of the cognate transcription factors. At present, it is unclear whether any of the reported mammalian proteins represents the physiological partner for YAP. Along this line, it is worth noting that while the reported ability of YAP to transactivate p73 in cultured mammalian cells is more suggestive of a tumor suppressor function for YAP (Basu et al., 2003), our studies clearly implicate Yki and YAP as potential oncogenes. One interesting possibility is that the reported coupling of mammalian YAP to p73 might represent a fail-safe mechanism to limit the oncogenic potential of YAP in much the same way as cell death is obligatorily linked to oncogene activation (Lowe et al., 2004).

An important direction in the future is to identify the DNA binding transcription factor (denoted as "X" in Figure 8) that partners with Yki to regulate gene transcription; identifying the factor should provide critical insights into how Yki (and likely YAP as well) could function as a potent oncogene. This effort should be facilitated by the dissection of the *diap1* promoter and the identification of a minimal Hpo-responsive element that confers transcriptional regulation of *diap1* by the Hpo pathway. With such a DNA element, one should be able to identify the DNA binding transcription factor that partners with Yki to regulate the transcription of *diap1* and other Hpo-pathway-responsive genes.

A Conserved Role for the Hpo Pathway in Mammalian Growth Control and Tumorigenesis?

Many components of the Hpo pathway are conserved throughout evolution, suggesting that this emerging pathway might play a similar role in mammals. Indeed, previous studies have shown that human homologs of *wts*, *hpo*, and *mats* could rescue the respective *Drosophila* mutants (Tao et al., 1999; Wu et al., 2003; Lai et al., 2005). Moreover, mice lacking a *wts* homolog are prone to tumor formation (St John et al., 1999), and the human orthologs of *sav* and *mats* are mutated in several cancer cell lines (Tapon et al., 2002; Lai et al., 2005). Such conservation is further extended in the current study, showing that Yki and YAP have similar biological activity when assayed in *Drosophila*. These results suggest that the Hpo signaling pathway might play a conserved role in mammalian growth control. Furthermore, inactivation of growth suppressors of the Hpo pathway, such as Hpo, Sav, Wts, and Mats, and hyperactivation of growth promoters of the pathway, such as YAP, are likely to contribute to mammalian tumorigenesis.

Experimental Procedures

Gene Targeting and Rescue of *yki*

The ends-out gene targeting strategy (Gong and Golic, 2003) involves cloning DNA segments flanking the target locus into a specially designed vector containing a w^{hs} marker gene between the homologous DNA segments, as well as features that allow the generation of a linear DNA template for homologous recombination

with the target locus following the action of FLP recombinase and I-SceI endonuclease. To construct *yki* knockout construct, two pairs of oligos, 5′-AGCAGGCGCGCCAATGTATACATCTGTATTAG ACC-3′ and 5′-AGCAGGCGCGCCCTTACAAAACTTTTGCCACTG-3′, and 5′-AGCAGCGGCCGCGGGGTGTTAGTAGCTTCAGGTT-3′ and 5′-AGCAGCGGCCGCATCTTAGCGATTAGGCACGCGCAC-3′, were used to amplify DNA fragments of ~4 kb from the BAC clone 27M17. These fragments were cloned into the AscI and NotI sites of pW25, respectively. This targeting construct is expected to replace all the coding sequence of *yki* (19573700–19575921 of the *Drosophila* genome sequence) with w^{hs}. Flies carrying the targeting construct on the 3rd chromosome were crossed to flies carrying the FLP and I-SceI enzymes, and the progeny were screened for precise gene targeting as described (Gong and Golic, 2003).

yki rescue construct was made by cloning a full-length *yki* cDNA downstream from the α-tubulin promoter. Multiple insertions of the *tub-yki* construct were tested for their ability to rescue three independently derived *yki* knockout alleles. All transgene/mutant combinations resulted in complete rescue.

Yeast Two-Hybrid Screen

Yeast two-hybrid was carried out using Stratagene's CytoTrap system, which is based on the ability of human Sos to complement a temperature-sensitive *cdc25* allele (*cdc25H*) in yeast when Sos is targeted to the plasma membrane through bait-prey interactions (Aronheim et al., 1997). In this system, the bait protein is expressed as a fusion protein with human Sos. The prey library contains cDNA clones fused with a myristylation signal that targets proteins to the cell membrane. Expression of the prey is further controlled by the GAL1 promoter, which is induced on galactose but repressed on glucose medium. When the bait and the cDNA library are cotransformed into the *cdc25H* strain, the only cells capable of growing at restrictive temperature on galactose medium are those that have been rescued by bait-prey interactions that recruit Sos to the cell membrane.

The three *yki* clones recovered from the yeast screen differ in their N termini but are otherwise identical, all including the termination codon and polyA. The Berkeley *Drosophila* Genome Project has sequenced a cDNA (LD21311) from the *yki* locus. LD21311 represents a differentially spliced form of *yki* and differs from our yeast two-hybrid positives in that the former contains only one WW domain. Compared to the two-WW form, the one-WW form of Yki has much reduced activity in overexpression and rescue assays (J.H., unpublished data). To construct a full-length *yki* cDNA with two WW domains, a BamHI-XhoI fragment of LD12311 was replaced with the corresponding fragment from the yeast two-hybrid positives. The resulting *yki* cDNA was used in all subsequent studies.

Drosophila Strains

All crosses were done at 25°C unless otherwise indicated. The following flies have been described: GMR-Hpo (Wu et al., 2003), GMR-Wts (Tapon et al., 2002), hpo$^{42-47}$ (Wu et al., 2003), sav^3 (Tapon et al., 2002), wts^{X1} (Xu et al., 1995), thi^{5c8} (Hay et al., 1995), cycE-lacZ (Jones et al., 2000). *yki* cDNA was cloned into pUAST and pGMR, and a human YAP cDNA (IMAGE clone 5747370) was cloned into pUAST to generate UAS-YAP.

FLP/FRT was used to generate mutant clones in imaginal discs. For double mutant clones of *hpo*, *sav*, or *wts* with *yki*, the following genotypes were used. Note that a tub-yki insertion on 3R was used in generating *sav yki* and *wts yki* double mutant clones.

hpo yki double mutant clones:
y w hsp-flp; FRT42D hpo$^{42-47}$ yki^{B5}/FRT42D Ubi-GFP
sav yki double mutant clones:
y w hsp-flp; FRT42D yki^{B5}/FRT42D yki^{B5}; FRT82B sav^3/FRT82B Ubi-GFP P[tub-yki]
wts yki double mutant clones:
y w hsp-flp; FRT42D yki^{B5}/FRT42D yki^{B5}; FRT82B wts^{X1}/FRT82B Ubi-GFP P[tub-yki]

S2 Cell Assays

HA-Yki was constructed by adding a C-terminal HA tag (YPYDVP-DYA) using the pAc5.1/V5-HisB vector (Invitrogen). N-terminal V5-tagged Wts was constructed in the same vector by adding a V5

tag (GKPIPNPLLGLDST) between the first and second codons of Wts. Point mutations of Yki and Wts were introduced using the QuikChange mutagenesis kit (Stratagene). Myc-tagged Hpo and Flag-tagged Sav have been described (Wu et al., 2003). Full length Yki was cloned into pGEX4T-1 to express a GST-Yki fusion protein. Our GST-Yki preparation contained two Yki-related bands, with the upper band migrating at the expected molecular weight. It also contained a contaminating band (Figure 6C). The Yki-related bands, but not the contaminating band, were diminished by thrombin, which cleaves the GST-Yki fusion at the junction of GST and Yki (data not shown). GST-Tsc1 fusion has been described (Wu et al., 2003). Transfection, immunoprecipitation, and in vitro kinase assay were carried out as described (Wu et al., 2003).

An in-frame fusion of the DNA binding domain (DB) of Gal4 and full-length Yki was constructed in pActGal4(1-147)SK (a gift of Albert Courey). The resulting plasmid, pActGal4DB-Yki, was transfected in triplicates with G5-37tkluc, a Gal4-responsive luciferase reporter (a gift of Albert Courey), with or without plasmids expressing Hpo, Sav, and Wts. Luciferase assay was carried out using Luciferase Assay System (Promega) and a FLUOstar luminometer (BMG LabTechnologies).

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Accession Numbers