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Tao-1 Phosphorylates Hippo/MST Kinases to Regulate the Hippo-Salvador-Warts Tumor Suppressor Pathway

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SUMMARY

Recent studies have shown that the Hippo-Salvador-Warts (HSW) pathway restrains tissue growth by phosphorylating and inactivating the oncoprotein Yorkie. How growth-suppressive signals are transduced upstream of Hippo remains unclear. We show that the Sterile 20 family kinase, Tao-1, directly phosphorylates T195 in the Hippo activation loop and that, like other HSW pathway genes, *Tao-1* functions to restrict cell proliferation in developing imaginal epithelia. This relationship appears to be evolutionarily conserved, because mammalian Tao-1 similarly affects MST kinases. In S2 cells, Tao-1 mediates the effects of the upstream HSW components Merlin and Expanded, consistent with the idea that Tao-1 functions in tissues to regulate Hippo phosphorylation. These results demonstrate that one family of Ste20 kinases can activate another and identify Tao-1 as a component of the regulatory network controlling HSW pathway signaling, and therefore tissue growth, during development.

INTRODUCTION

During development, organisms must determine the overall size and shape of their individual organs through mechanisms not fully understood. The recent discovery of the evolutionarily conserved Hippo-Salvador-Warts (HSW) signaling pathway has revealed a unique mechanism to regulate proliferation independent of developmental patterning. The core members of the HSW pathway, Hippo (Hpo) and Warts (Wts), together with their scaffolding partners Salvador (Sav) and Mats, phosphorylate and inactivate the transcriptional coactivator Yorkie (Yki; reviewed in Edgar [2006] and Pan [2007]). Phosphorylation prevents Yorkie from translocating to the nucleus where it binds to TEAD-family transcription factors and drives the transcription of genes that promote growth and inhibit apoptosis (Huang et al., 2005). Loss of HSW pathway function in *Drosophila* leads to increased cellular proliferation resulting in tumor-like overgrowths in epithelial tissues. Similarly, knockout mouse models of HSW homologs grow tumors, and human HSW homologs have been implicated in cancers (Pan, 2010; Zhao et al., 2010). These studies suggest that HSW signaling is a crucial part of an organism's ability to regulate cell proliferation and overall tissue size.

A central, unanswered question regarding HSW function is how the activity of Hpo, the most upstream kinase in the pathway is regulated. The atypical cadherin Fat and its ligand Dachsous can function at the plasma membrane to initiate HSW signaling, however the extracellular cues that trigger signaling and the mechanism by which Fat activates Hpo remain elusive (Bennett and Harvey, 2006; Silva et al., 2006; Willecke et al., 2006). In addition, genetic evidence strongly suggests that another source of Hpo activation functioning in parallel to Dachsous-Fat activation must exist (Cho et al., 2006). At least three different cytoplasmic proteins are believed to act upstream of Hpo to initiate signaling through the pathway, Expanded (Ex), Merlin (Mer), and Kibra (Hamaratoglu et al., 2006; Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010). Ex and Mer are members of the four-point-one, ezrin, radixin, moesin (FERM) family and Kibra is a WW-domain containing protein. Though these three proteins are thought to physically interact with each other in varying complexes (McCartney et al., 2000; Genevet et al., 2010; Yu et al., 2010), only Ex can form a complex with Hpo (Yu et al., 2010) and it is unclear how this interaction leads to activation of Hpo. Moreover, there is strong genetic evidence that Ex, Mer, and Kibra act in parallel to each other (Hamaratoglu et al., 2006; Maitra et al., 2006; Genevet et al., 2010), implying that other mechanisms for activating Hpo independently of Ex must exist.

We sought to identify genes that might function upstream of Hpo to activate the pathway using a candidate gene approach and discovered that the Sterile 20 kinase Tao-1 is a member of this signaling pathway. Tao-1 previously has been shown to destabilize microtubules and has been implicated in apoptosis in the *Drosophila* germline (Sato et al., 2007; Liu et al., 2010). Here, we show that loss of *Tao-1* function results in increased cellular proliferation and upregulation of Yki target gene expression. We further demonstrate that Tao-1 regulates HSW pathway activity by phosphorylating Hpo at a critical activating residue. Thus, these results identify Tao-1 as a member of the HSW pathway and provide a molecular mechanism for Hpo activation.

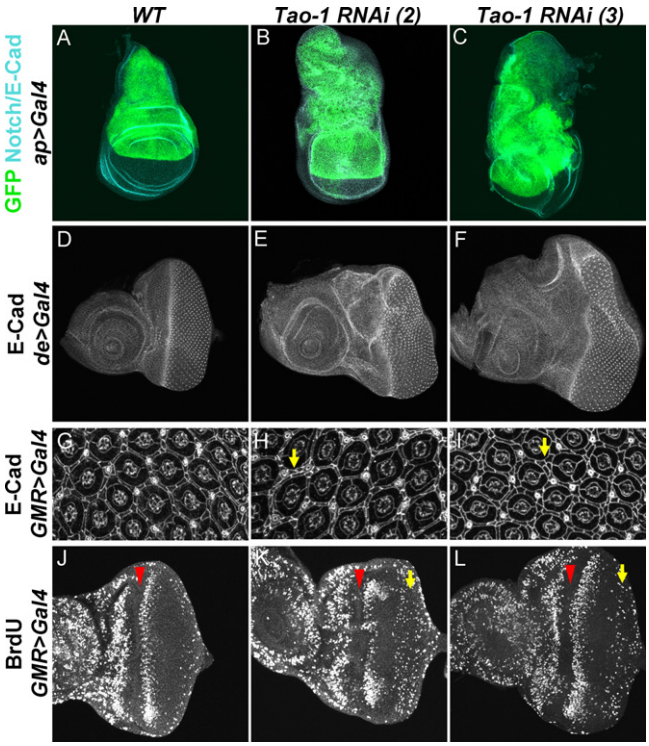

Figure 1. Loss of Tao-1 Function Causes Overproliferation

(A–C) *ap>Tao-1 RNAi* causes massive overgrowth of 3rd instar wing imaginal discs compared to wild-type discs. GFP marks the *apterous* domain, and Notch or E-Cadherin is in blue. Ventral is down.

(D–F) *Tao-1* knockdown causes overproliferation in the eye imaginal disc. Larval eye imaginal discs expressing *Tao-1 RNAi* under the control of the *dorsal enhancer* driver (*de>Tao-1 RNAi*) are overgrown dorsally compared to wild-type eye discs. Anterior is to the left and ventral down in all eye disc images.

(G–I) E-cadherin staining of pupal eyes 43 hr APF showing increased numbers of interommatidial cells in *GMR>Tao-1 RNAi* eyes compared to *GMR-Gal4/+* eyes. Yellow arrows point out regions where interommatidial cell layer is doubled.

(J–L) Representative images of *GMR-Gal4/+* and *GMR>Tao-1 RNAi* third instar eye discs labeled with BrdU to indicate mitotically active cells. Note increased incorporation of BrdU posterior to the second mitotic wave (yellow arrow) in *GMR>Tao-1 RNAi* discs. The morphogenetic furrow is marked by the red arrowhead. *Tao-1 RNAi* transgenes on the second chromosome (images in B, E, H, K) and third chromosome (images in C, F, I, L) give similar phenotypes. Unless otherwise noted, all subsequent experiments using *Tao-1 RNAi* were conducted with the transgene on the third chromosome, which was used primarily for convenience in building strains.

RESULTS

Tao-1 Has Tumor Suppressor Functions

Recent work has demonstrated that there are multiple, parallel inputs into the HSW pathway, but the molecular mechanisms that regulate Hpo activation remain unclear. Given that two Sterile 20 family kinases, Hpo and Slik (Harvey et al., 2003; Hughes and Fehon, 2006), have been implicated in HSW signaling, we wondered if other family members might be involved in upstream regulation of this growth control pathway. Using RNA interference in the wing imaginal disc, we tested the remaining nine Sterile 20 kinases in the *Drosophila* genome (see Figure S1A available online) for effects on growth and HSW target gene expression. Of these, only *Tao-1* emerged as a strong regulator of growth and HSW pathway output.

Compared to wild-type controls, the dorsal compartment of wing imaginal discs from animals expressing *Tao-1 RNAi* under the control of the *apterous-Gal4* driver (*ap>Tao-1 RNAi*) was larger than normal and misshapen (Figures 1A–1C). In adult wings from animals expressing *Tao-1 RNAi* under the *decapentaplegic-Gal4* driver (*dpp>Tao-1 RNAi*), extra folds and vein material were present (Figures S1B and S1C). Quantifying the area between veins three and four of these wings revealed a statistically significant increase in size relative to wild-type wings ($p < 0.001$; Figure S1D). Using a driver specific for the dorsal compartment of the eye (*de-Gal4*), we also noted an increase in the size of eye imaginal disc tissue lacking *Tao-1* function. *de>Tao-1 RNAi* eyes had grossly overgrown dorsal compartments relative to both the ventral compartment and to wild-type controls (Figures 1D–1F). The increased size in eye imaginal discs can be attributed to increased cell number, as demonstrated by the higher number of interommatidial cells in pupal eyes from *GMR>Tao-1 RNAi* flies (Figures 1G–1I). These cells are normally eliminated through apoptosis during early pupal development (Wolff and Ready, 1991), but persist in animals mutant for components of the HSW pathway, suggesting that *Tao-1* may function in the HSW pathway.

HSW pathway mutants are characterized by inappropriate persistence of cell division in the developing third instar eye imaginal disc. In a wild-type eye disc, cells become quiescent after a final synchronous round of cell division (termed the second mitotic wave [SMW]) following the passing of the morphogenetic furrow, which moves anteriorly across the eye disc to initiate differentiation and patterning. In animals lacking *hpo* or *wts* function, cells fail to exit the cell cycle and undergo division after the SMW, shown by BrdU incorporation during S phase (Harvey et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003). In *GMR>Tao-1 RNAi* eyes, but not in controls, BrdU incorporation occurs posterior of the SMW (Figures 1J–1L). This suggests that the defect in animals lacking *Tao-1* function may be related to the defect observed in HSW signaling mutants.

Two lines of evidence verify the identity and specificity of the RNAi transgenes used in these experiments. First, two nonoverlapping *Tao-1 RNAi* transgenes (Dietzl et al., 2007) were tested and gave similar phenotypes (Figure 1). Second, these transgenes reduced antibody staining of endogenous Tao-1 (Figures S1E–S1E′). Taken together, these results indicate that these transgenes specifically knockdown Tao-1 expression.

Previous studies have identified three alleles of *Tao-1*, which is located very proximally (18D) on the X chromosome, but it has not been possible to test their effects on growth. Sato et al. (2007) described a null allele that was lethal and sterile in germline clones, but this allele has been lost (Kobayashi, personal communication). King et al. (2011) recently described two alleles. One is associated with a 2 kb deletion at the 5′ end that appears to affect an adjacent gene ($Tao\text{-}1^{50}$), and the other is viable and only slightly affects Tao-1 expression ($Tao\text{-}1^{EP1455}$). Unfortunately, $Tao\text{-}1^{50}$ mutants die at the second larval instar, precluding any analysis of overgrowth in imaginal discs (data not shown).

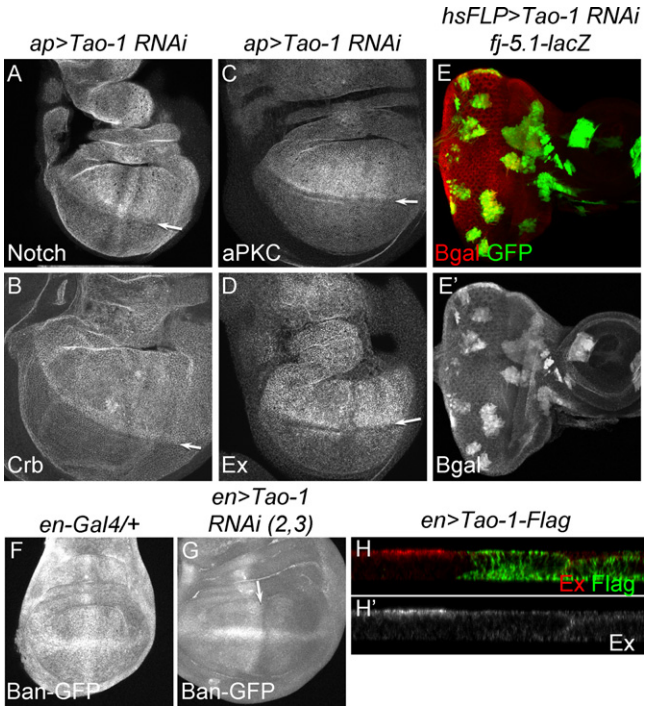

Figure 2. *Tao-1* Depletion Causes Upregulation of Many Known Targets of the HSW Pathway

(A–D) *ap>Tao-1 RNAi* results in upregulation of known HSW targets within the dorsal compartment of the wing. (A) Notch, (B) Crumbs, (C) aPKC, and (D) Expanded are apically enriched in *Tao-1 RNAi* wing discs. Arrows mark the D-V boundary.

(E and E′) In the eye imaginal disc, *four-jointed-LacZ*, a transcription reporter for HSW signaling, is upregulated in heat-shock flip-out cell clones expressing *Tao-1 RNAi* (marked by GFP).

(F and G) In the wing, *Tao-1* knockdown under the *en-Gal4* driver results in increased activity of the *bantam* miRNA (as assayed by the bantam-GFP sensor, Brennecke et al., 2003), a *yki* target gene, suggesting increased *bantam* transcription. Arrow indicates A-P boundary.

(H and H′) Expression of Flag-tagged *Tao-1* under the *engrailed* promoter (*en>Tao-1-Flag*) results in lower Ex levels in the posterior compartment of wing discs. In all images ventral is down and posterior to the right.

Loss of *Tao-1* Causes Upregulation of HSW Pathway Targets

Another hallmark of defects in HSW signaling is an increase in levels of cell surface proteins including receptors and adhesion proteins, as well as juxtamembrane proteins involved in cell polarization and intracellular signaling. We found elevated levels of Notch, Crumbs, and aPKC (Figures 2A–2C), as well as phospho-moesin, E-cadherin, and PatJ (Figures S2D–S2F) in *ap>Tao-1 RNAi* wing discs. Proteins that act as upstream activators of the HSW pathway are also transcriptional targets of the pathway and are upregulated apically in *hpo* or *wts* mutant cells (Hamaratoglu et al., 2006; Genevet et al., 2010). Consistent with this, we found increased apical levels of Ex, Mer, Fat, and Kibra (Figure 2D and Figures S2A–S2C) in *ap>Tao-1 RNAi* wing discs.

To confirm that loss of *Tao-1* results in transcriptional upregulation of known HSW target genes, we examined the expression of lacZ transcriptional reporters for *four-jointed* and *ex* (Cho et al., 2006; Hamaratoglu et al., 2006). In the absence of *Tao-1* function, transcription of *four-jointed* and *ex* was increased in eye and wing imaginal discs, respectively (Figures 2E–2E′ and Figures S2G–S2G′). Activity of another HSW target gene, the miRNA *bantam,* was also higher (as assayed by a GFP bantam reporter; Brennecke et al., 2003) in tissues lacking *Tao-1*, suggesting elevated levels of *bantam* (Figures 2F and 2G). Overexpression of Tao-1 lowered levels of Ex at the apical surface, consistent with overexpression of other HSW pathway genes (Figures 2H–2H′). These results are similar to those of known HSW pathway genes, suggesting that *Tao-1* is also a member of this signaling pathway.

Tao-1 Functionally Interacts with Members of the HSW Pathway and Promotes Wts Phosphorylation

Given that *Tao-1* loss-of-function phenotypes are similar to HSW pathway mutants, we asked whether *Tao-1* genetically interacts with any of the previously identified HSW pathway genes. Previous studies in *Drosophila* have shown that *wts* overexpression can rescue the lethality of *fat* and *ex* null mutations (Feng and Irvine, 2007). Similarly, we found that coexpression of *wts* in the wing strongly suppressed the lethality of *Tao-1 RNAi* driven by *engrailed-Gal4* (*en>Tao-1 RNAi)*, suggesting that *Tao-1* functions through HSW signaling (Figure 3A). Additionally, while *en>wts* wings appeared under proliferated, having smaller posterior compartments than *en-Gal4* controls, this under proliferation was partially suppressed in *en>wts; Tao-1 RNAi* wings (Figures 3B–3D). This result suggests that complete activation of Wts in the wing requires Tao-1 activity. Taken together, these results suggest that *Tao-1* is required for complete HSW pathway activation in developing epithelial tissues.

Wts activity is regulated by the upstream kinase Hpo, and Wts phosphorylation is a commonly used readout of HSW pathway activation (Huang et al., 2005; Dong et al., 2007). We next asked whether Tao-1 promotes Wts phosphorylation in *Drosophila* S2 cells using a phospho-specific Wts antibody that recognizes a critical regulatory residue (T1077; Yu et al., 2010). We found that Wts phosphorylation was substantially increased in the presence of Tao-1 and that expression of a kinase-dead form of Tao-1 strongly reduced Wts phosphorylation (Figure 3E). Thus, like several other upstream activators of HSW signaling Tao-1 regulates pathway activation by modulating Wts phosphorylation.

Most, if not all, HSW pathway function is dependent on the transcriptional coactivator Yki, which is thought to promote growth through target genes such as *Cyclin E*, *DIAP1*, and the miRNA *bantam* (Huang et al., 2005; Nolo et al., 2006; Thompson and Cohen, 2006). In addition to these growth-promoting genes, Yki also regulates expression of the tumor suppressor genes *ex*, *Mer*, and *kibra* as part of a negative feedback loop (Hamaratoglu et al., 2006; Genevet et al., 2010). To ask if overgrowth and Ex upregulation in cells lacking *Tao-1* function is Yki-dependent, we generated flies expressing both *yki* and *Tao-1 RNAi* and found that both overproliferation and Ex staining were suppressed (Figures 3F–3H′). In fact, *en>yki RNAi* and *en>yki RNAi; Tao-1 RNAi* wing discs were nearly indistinguishable from one other, indicating that Tao-1 functions through Yki to regulate organ size.

Tao-1 Phosphorylates Hpo to Activate the HSW Pathway

We next sought to determine where Tao-1 functions within the HSW pathway using a combination of biochemical assays in S2 cells and genetic epistasis experiments in vivo. Because the

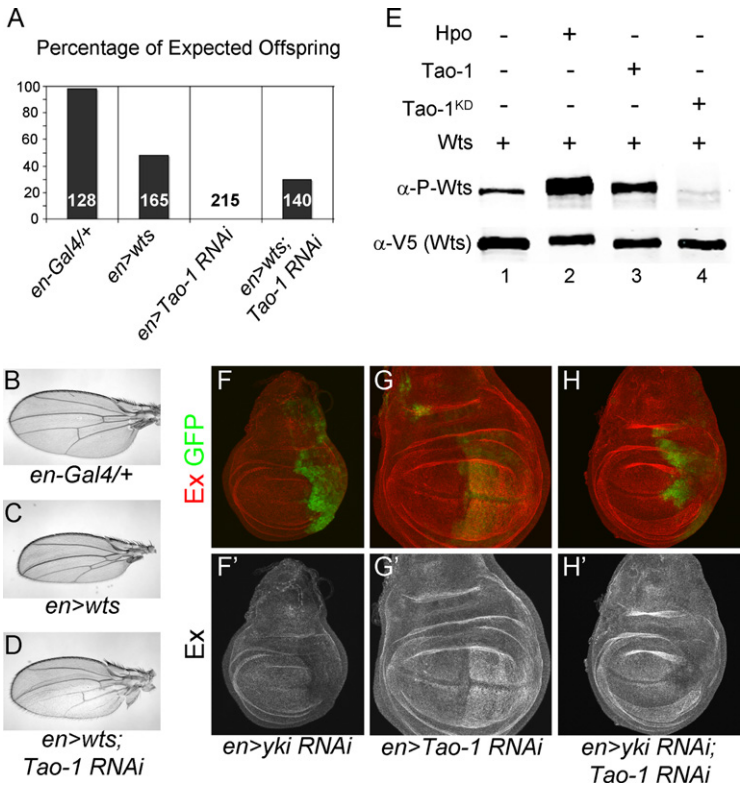

Figure 3. *Tao-1* Functionally Interacts with HSW Pathway Components

(A) Lethality caused by loss of *Tao-1* is suppressed by expressing a *wts* transgene. Surviving adults were scored 1–2 days after eclosion and the percentage of expected offspring was plotted for each genotype. The number within each bar represents the expected number of adult progeny for each genotype.

(B–D) *Tao-1 RNAi* suppresses the effect of Wts overexpression in the wing.

(B and C) Male *en-Gal4/+* control wings appear normal, whereas *en>wts* wings have a smaller posterior compartment.

(D) *en>wts; Tao-1 RNAi* animals survive to adults and have an intermediate wing phenotype.

(E) Tao-1 promotes Wts phosphorylation in *Drosophila* S2 cells. Tao-1 and Wts were transiently expressed in S2 cells and Wts phosphorylation was measured using a phospho-specific antibody (Yu et al., 2010). Levels of Wts phosphorylation (lane 1) were increased in the presence of Hpo and Tao-1 (lanes 2 and 3). In contrast, Wts phosphorylation was severely diminished in the presence of a kinase-dead form of Tao-1 (lane 4).

(F–H′) HSW pathway target gene upregulation in *Tao-1 RNAi* knockdown requires *yki*.

(F–G′) Reduction of *yki* using the *en-Gal4* driver leads to a reduction in Ex staining, whereas *Tao-1 RNAi* results in increased Ex expression.

(H and H′) Double knockdown of *Tao-1* and *yki* causes decreased levels of Ex, similar to *yki RNAi* alone.

Sterile 20 kinase Hpo is known to phosphorylate Wts (Wu et al., 2003), we looked at whether Hpo is required for Tao-1 to promote Wts phosphorylation. We used double stranded RNA (dsRNA) to deplete S2 cells of endogenous Hpo and found that Tao-1 required Hpo to promote Wts phosphorylation (Figure 4A). This strongly suggested that Tao-1 functions upstream of Hpo. To test this hypothesis, we next asked whether Tao-1 promotes Hpo phosphorylation in S2 cells. For these experiments we used a kinase-dead form of Hpo (Hpo^{KD}) to prevent auto-phosphorylation, which could obscure Tao-1 effects on Hpo phosphorylation. We found that levels of Hpo^{KD} phosphorylation were increased in the presence of wild-type, but not kinase-dead Tao-1. Furthermore, knockdown of *Tao-1* using dsRNA decreased levels of Hpo^{KD} phosphorylation (Figure 4B). These results suggest that Tao-1 functions through Hpo to modulate pathway activity and that endogenous Tao-1 is required for normal levels of Hpo phosphorylation found in unstimulated S2 cells.

Previous studies have shown that overexpression of Hpo results in decreased levels of Ex in the imaginal epithelia (Hamaratoglu et al., 2006). If Tao-1 functions upstream of Hpo, then overexpression of wild-type Hpo should suppress the upregulation of Ex in *Tao-1 RNAi* wing discs. To test this hypothesis, we simultaneously expressed *UAS-hpo* and *Tao-1 RNAi* in the wing using the *dpp>Gal4* driver. These crosses were done at 18°C to prevent excessive tissue loss resulting from Hpo-induced apoptosis. We found that *dpp>hpo* wing discs were indistinguishable from *dpp>hpo; Tao-1 RNAi* discs and that both had decreased levels of Ex staining, whereas *dpp>Tao-1 RNAi* discs had elevated levels of Ex (Figures 4C–4E). Additionally, in the reciprocal experiment we found that overexpression of Tao-1 could not suppress the upregulation of Ex resulting from reduction in *hpo* function (data not shown). We conclude from these results that Tao-1 functions upstream of Hpo to regulate cell proliferation and target gene expression.

Our observation that Hpo phosphorylation in S2 cells is Tao-1-dependent suggests that Hpo could be a direct substrate for Tao-1 kinase activity. To test this hypothesis we examined the ability of purified Tao-1 to phosphorylate recombinant Hpo protein in vitro. In control experiments using P32 and autoradiography, we found that full-length Tao-1 immunoprecipitated from S2 cells was able to autophosphorylate and therefore was catalytically active. In this experiment we failed to detect any other copurifying autocatalytic kinases, suggesting that Tao-1 does not copurify with an intermediary kinase (Figure S3A). We then tested the ability of purified Tao-1 to phosphorylate the isolated kinase domain of Hpo (GST-$Hpo^{kin\ dom}$), which contains the activation loop with a critical regulatory residue (T195) that controls Hpo activity (Colombani et al., 2006). Using an antibody specific for phosphorylation at T195, we found that purified Tao-1 clearly phosphorylated Hpo at this site (Figure 4F, lane 2). This phosphorylation was specific because it was not observed with a kinase dead form of Tao-1 (Figure 4, lane 1), and a mutation in the activation loop that blocks Hpo activation, T195A, completely blocked this signal (Figure 4F, lanes 3–4). Additional in vitro kinase experiments using P32 confirmed that Tao-1 directly phosphorylates Hpo (Figure S3A). These results indicate that Tao-1 directly activates Hpo by phosphorylating at a critical regulatory site.

To further confirm the specificity of Hpo as a substrate for Tao-1 we next asked whether a mammalian ortholog of Tao-1, TAOK3, was able to directly phosphorylate MST1 and 2, the mammalian orthologs of Hpo. Interestingly we found that human TAOK3, which shares 70% identity with the kinase domain of

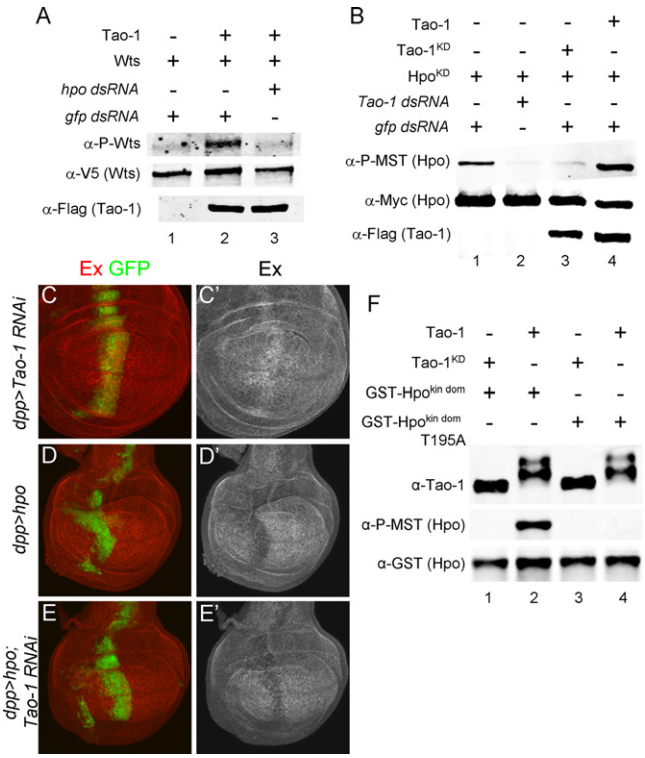

Figure 4. Tao-1 Phosphorylates Hpo to Activate the HSW Pathway

(A) Hpo is required for Tao-1 to promote Wts phosphorylation in S2 cells. Normally, Wts is phosphorylated in the presence of Tao-1 (lane 2); however, transfecting *hpo* dsRNA prevents Wts phosphorylation (lane 3).

(B) Tao-1 promotes Hpo phosphorylation at a critical regulatory residue (T195). Cells were transfected with a kinase-dead form of Hpo (Hpo^{KD}) to prevent autophosphorylation. A low level of Hpo phosphorylation was detected in these cells using a commercially available phospho-MST1/2 antibody (lane 1), which specifically recognizes phosphorylation at T195. This level of phosphorylation can be diminished by depleting endogenous Tao-1 (lane 2), or by expressing a kinase-dead form of Tao-1 ($Tao\text{-}1^{KD}$; lane 3). Hpo phosphorylation is greatly increased in the presence of wild-type Tao-1 (lane 4).

(C–E′) *Tao-1* functions through *hpo* to regulate HSW pathway target genes.

(C and C′) As shown previously, loss of *Tao-1* results in increased Ex levels.

(D and D′) *UAS-hpo* expressed using a *dpp-Gal4* driver (*dpp>hpo*) results in decreased Ex expression.

(E and E′) *dpp>hpo; Tao-1 RNAi* also results in decreased Ex staining, suggesting that *hpo* functions downstream of *Tao-1*. All flies raised at 18°C.

(F) Tao-1 directly phosphorylates T195 within the activation loop of Hpo. Flag-tagged Tao-1 was immunoprecipitated from S2 cells and incubated with recombinant Hpo kinase domain (aa 1–367; $GST\text{-}Hpo^{kin\ dom}$). Immunoprecipitated Tao-1 displayed a mobility shift (lanes 2 and 4) relative to $Tao\text{-}1^{KD}$ (lanes 1 and 3), indicating that Tao-1 was catalytically active. Staining with phospho-MST1/2 antibody revealed that Tao-1 phosphorylates the kinase dead mutant of Hpo at T195 (lane 2). The T195A mutation confirmed that the phospho-MST1/2 antibody specifically recognizes phosphorylation at T195 (lane 4).

Tao-1, was able to directly phosphorylate the activation of loop of human MST1 and 2 in vitro (Figure S3B). This result strongly suggests that the role of Tao-1 in HSW signaling is functionally conserved in mammals.

Merlin and Expanded Function with Tao-1 to Activate HSW Signaling

The cytoplasmic proteins Mer and Ex have been reported to promote HSW pathway activation (Hamaratoglu et al., 2006), so we next asked if Tao-1 mediates their effect on HSW activation in cultured cells. As a readout of HSW activation, we examined phosphorylation of Wts, which is increased by expression of Mer and Ex (Yu et al., 2010; Figure 5A, lane 2). This effect is substantially blocked by cotransfection with dsRNA against *Tao-1* (Figure 5A, lane 3; controls for Tao-1 dsRNA knockdown efficiency are shown in Figure S4B), suggesting that Tao-1 mediates the effects of Mer and Ex on HSW signaling. Conversely, we found that activation of Wts by Tao-1 was not affected by depletion of Mer and Ex (Figure 5B), suggesting that Tao-1 functions downstream of these proteins. We also found that depletion of Mer or Ex had no effect on the ability of Tao-1 to promote Hpo phosphorylation (Figure 5C), further arguing that Tao-1 functions independently of Mer and Ex (the converse experiment was not possible because we were unable to detect an effect of Mer and Ex expression on Hpo phosphorylation state, data not shown). A similar absence of effect was seen with depletion of Kibra (Figure 5C), but we were unable to unambiguously test whether Wts phosphorylation induced by Kibra is Tao-1 dependent because in our experiments expression of Kibra resulted in greatly decreased Wts expression. Attempts to coimmunoprecipitate Flag-tagged Tao-1 from S2 cells with Mer, Ex or Kibra proved unsuccessful (data not shown), suggesting that these proteins do not form a complex.

DISCUSSION

In an effort to identify additional regulators of HSW signaling we have examined the role of Tao-1 in growth control during development. Tao-1 depletion in either the eye or wing epithelium results in overgrowth phenotypes as well as transcriptional upregulation of HSW targets. Using a combination of genetic epistasis, experiments in cultured S2 cells, and in vitro biochemistry, we have demonstrated that Tao-1 directly phosphorylates the critical T195 regulatory residue in the activation loop of Hpo to promote HSW pathway activation. The observation that a mammalian ortholog of Tao-1, TAOK3, can phosphorylate MST kinases at the same residue further suggests that this regulatory function is conserved in mammals. Taken together, these results implicate Tao-1 as a component of HSW signaling and reveal a mechanism for regulation of Hpo activity (Figure 6).

Although *Tao-1* depletion results in overgrowth phenotypes that are similar to mutations in other HSW pathway genes, these phenotypes are less severe than those of core components such as *hpo* and *wts*. One likely explanation for this is that the RNAi transgenes we have used in these studies do not completely remove *Tao-1* function. It is also possible that there are multiple mechanisms for activating HSW signaling, including, but not limited to, Tao-1 phosphorylation of Hpo. Indeed, previous studies have demonstrated that the upstream components Mer, Ex, and Kibra act, at least in part, in parallel to activate Hpo (Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010). Our biochemical evidence indicates that two of these proteins, Mer and Ex, function with Tao-1 to activate HSW signaling. Although it is probable that Kibra functions upstream of Tao-1, we cannot rule out the possibility that Kibra functions independently of Mer and Ex to activate HSW signaling in a Tao-1-independent manner (Figure 6). Further genetic analysis using a *Tao-1* null allele would be helpful in defining the role of Tao-1 relative to other HSW components, but unfortunately the

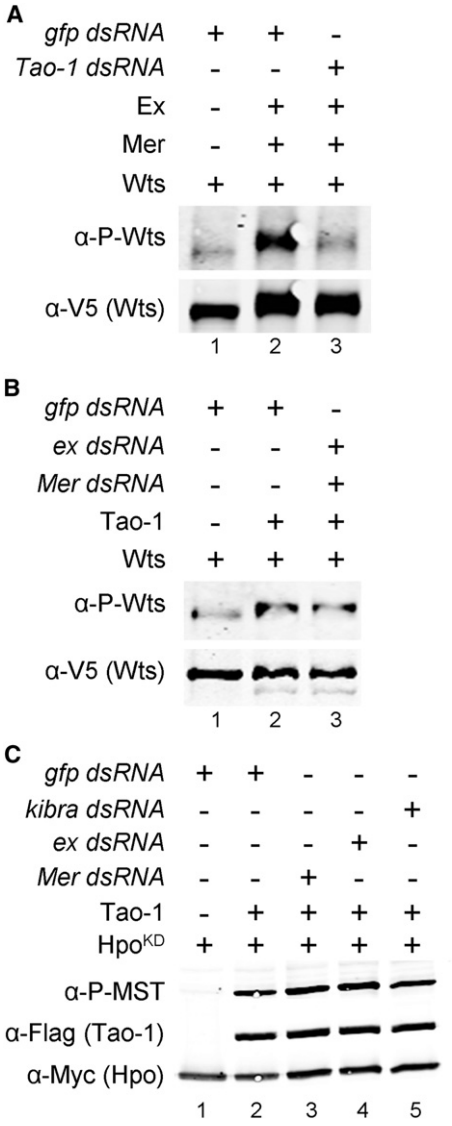

Figure 5. Mer and Ex Require Tao-1 to Activate the HSW Pathway
(A) Mer and Ex require Tao-1 to induce increased Wts phosphorylation. Normally Wts phosphorylation increases in the presence of Mer and Ex (lane 2). However, depleting Tao-1 with dsRNA eliminates this effect (lane 3).
(B) Tao-1 induced Wts phosphorylation is not dependent on Mer and Ex. Depletion of Mer and Ex by dsRNA (lane 3) does not affect Tao-1 induced p-Wts staining (lane 2).
(C) Tao-1 promotes Hpo phosphorylation independently of Mer, Ex and Kibra. S2 cells were transfected with dsRNA targeting *Mer* (lane 3), *ex* (lane 4), or *kibra* (lane 5). Knockdown of these genes had no effect on the ability of Tao-1 to promote Hpo phosphorylation (compare lane 2 to lanes 3–5).

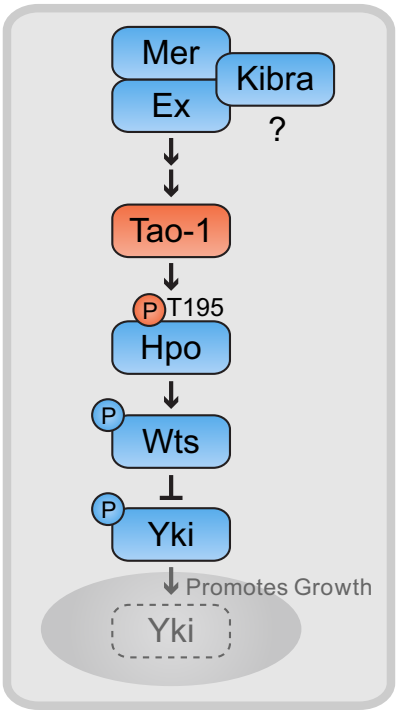

Figure 6. A Model for the Function of Tao-1 in the HSW Pathway
Tao-1 directly phosphorylates Hpo at T195 in the kinase activation loop, leading to the activation of Wts and inhibition of Yki, which remains cytoplasmic when phosphorylated. In the absence of HSW pathway activity, Yki translocates to the nucleus to promote transcription of target genes that promote growth and inhibit cell death. Genetic and biochemical experiments position Tao-1 upstream of Hpo and suggest that Mer and Ex function through Tao-1 to activate HSW signaling. Kibra, Mer, and Ex can form a protein complex and promote HSW activation, but it remains unclear if Kibra functions upstream or in parallel to Tao-1.

deletions associated with the sole existing *Tao-1* null allele, *Tao-1*^{50}, also appear to affect an adjacent gene (CG14218; King et al., 2011). In addition, *Tao-1* maps very close to the most proximal *FRT* element on the *X* chromosome, making it difficult to generate recombinant chromosomes for somatic mosaic analysis.

How do Mer, Ex, and Tao-1 cooperate to regulate Hpo phosphorylation? Given that Ex has been shown to interact with Hpo (Yu et al., 2010), one possibility is that Mer and Ex function to scaffold Tao-1 together with Hpo, thereby promoting the ability of Tao-1 to phosphorylate and activate Hpo. However, despite repeated attempts we have been unable to detect Tao-1 in a complex with either Mer or Ex, and knockdown of *Mer*, *ex*, or *kibra* does not diminish the ability of Tao-1 to promote Hpo phosphorylation in S2 cells. For these reasons, we favor the possibility that Mer and Ex indirectly affect Tao-1 function, perhaps by interacting with other proteins that in turn directly regulate Tao-1. For example, Tao-1 activity could be directly regulated by an unknown receptor at the cell surface whose localization or activity is controlled by interaction with Mer and Ex. This notion is consistent with the fact that both Mer and Ex have FERM domains, which are known to interact with the cytoplasmic tails of transmembrane proteins (Bretscher et al., 2002). Previous studies have suggested that Ex interacts with the transmembrane protein Crumbs (Ling et al., 2010), though the mechanistic significance of this interaction is unclear. It is not currently known whether *Drosophila* Merlin has transmembrane binding partners.

Two additional ideas related to Tao-1 function are suggested by our data. In S2 cells, Tao-1 kinase activity is required for normal levels of Hpo phosphorylation at T195 in the kinase activation loop, suggesting that Tao-1 could function to maintain constant, low levels of pathway activation. In turn, this low level of Hpo activation might be necessary so that other, regulated inputs into HSW activity can quickly transition cells away from

actively dividing and into a differentiated state following periods of growth. Alternatively, it is possible that Tao-1 activity itself is dynamically regulated during development, allowing it to rapidly alter levels of HSW pathway activity via its effect on Hpo phosphorylation. In either case, phosphorylation by Tao-1 at T195 is likely to promote the known ability of Hpo to undergo autophosphorylation (Colombani et al., 2006), thus amplifying the effect of even a small change in Tao-1 activity. Further studies will be required to answer these questions and to determine if, and how, Tao-1 activity is regulated.

An interesting aspect of our discovery that Tao-1 regulates HSW signaling is that Tao-1, and its mammalian orthologs TAOK1-3, previously have been shown to regulate MT stability (Mitsopoulos et al., 2003; Timm et al., 2003; Liu et al., 2010). Our results indicate that this effect on MT stability is not mediated through HSW signaling, because mutations in other HSW pathway components do not display similar MT phenotypes (data not shown). However, it is interesting to speculate that the association of Tao-1 with MTs might affect its ability to regulate HSW pathway activation. More work will be required to determine whether the function of *Drosophila* Tao-1 in HSW signaling is entirely independent of its role in microtubule dynamics, although a recent study in mammalian cultured cells found that microtubule disruption did not affect localization of Yap, a mammalian Yki ortholog (Dupont et al., 2011), suggesting that in mammalian cells these roles might be independent.

An additional possible mechanistic link between Tao-1 and HSW signaling is suggested by studies in flies and in mammalian cells indicating that Par-1, a polarity protein, is positively regulated by Tao-1 (Timm et al., 2003; King et al., 2011). Par-1 has been shown to promote basolateral polarity in the *Drosophila* follicular epithelium and to regulate the stability and organization of MTs in these cells (Doerflinger et al., 2003). Recent studies have implicated components of both apical and basolateral polarity in the regulation of HSW signaling (reviewed in Halder and Johnson, 2011). Conversely, HSW signaling also seems to feed back onto Crumbs, an apical determinant, and perhaps other components to regulate apical-basal polarity (Chen et al., 2010; Ling et al., 2010; Robinson et al., 2010). Whether Tao-1 plays a role in the linkage between cell polarity and growth control remains to be established, but the ability to both directly activate Hpo function through phosphorylation and control cytoskeletal organization and cell polarity through microtubule organization potentially places Tao-1 in a unique position to coordinate these important cellular processes.

EXPERIMENTAL PROCEDURES

Drosophila Genetics

All crosses were done at 25°C unless otherwise noted. We used the GAL4/UAS system throughout these experiments (Brand and Perrimon, 1993). Transgenic RNAi stocks were provided by the Vienna collection and include *Tao-1 RNAi (3)* (ID 17432), *Tao-1 RNAi (2)* (ID 107645), *hpo RNAi* (ID 104169), and *yki RNAi* (ID 104523) (Dietzl et al., 2007). *dpp>Tao-1 RNAi* wing area was measured using the AxioVision software (Zeiss) and compared to the average wing area of wild-type flies.

Immunofluorescence

Dissections and antibody staining were carried out as previously described (Maitra et al., 2006) with the following exceptions: For anti-Crb staining, discs were dissected in PBS and treated with methanol for 10 min following fixation. For BrdU incorporation, eye discs were dissected in 50 ug/ml BrdU in Ephrussi and Beadle's Ringers (4.7 mM KCl, 128 mM NaCl, 1.5 mM $CaCl_2$) within 10 min and incubated for a total of 30 min. Following one quick rinse in PBS with 0.1% Triton X-100 (PBST), discs were fixed in 3:1 EtOH/glacial acetic acid for 15 min, then rehydrated through a 75%, 50%, 25%, and 10% EtOH (in H_2O) series. Three rinses in PBST were followed by a 20 min treatment in 2N HCl and three more rinses in PBST. Finally, discs were incubated in PBST plus 0.1% NGS prior to antibody staining.

Fluorescent secondary antibodies were diluted to 1:1,000 (Jackson Immunoresearch Laboratories). Tissue samples were mounted in ProLong Antifade (Invitrogen). Confocal images were acquired on a laser-scanning confocal microscope (LSM510, Zeiss) using LSM software. The following objectives were used: 20× NA 0.8 Plan-Apochromat, 40X NA 1.3 EC Plan-NeoFluar and 63× NA 1.4 Plan-Apochromat. Images were compiled using Photoshop CS3 (Adobe).

Cell Culture and In Vitro Kinase Assays

Schneider-2 (S2) cell maintenance and transfection were performed as previously described (Neisch et al., 2010). Cells were lysed in lysis buffer containing 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 100 mM Tris pH 8.0, and protease inhibitors (Roche). Immunoblots were imaged using an Odyssey scanner with version 2.1 software (LI-COR Biosciences).

For RNAi knockdown experiments, T7-primers corresponding to nonconserved coding regions were used to generate 450–700 bp PCR products and dsRNA was transcribed from these templates using the MEGAscript High Yield Transcription Kit (Applied Biosystems).

For in vitro kinase assays, S2 cells expressing Flag-tagged Tao-1, Tao-1^{KD}, or TAOK3 were lysed in lysis buffer (see above) and immunoprecipitated with M2 anti-Flag agarose beads (Sigma-Aldrich), washed in PBS, and incubated with recombinant GST protein in kinase buffer containing 25 mM HEPES pH 7.2, 25 mM $MgCl_2$, 50 mM β-glycerol phosphate, 2 mM dithiothreitol, 0.5 mM sodium vanadate, 10 μM ATP, and 10 μCi/ml γ-^{32}P-ATP at 30°C for 40 min.

Expression Constructs

The kinase domains of Hpo (kinase dead), MST1 and MST2 consisting of amino acids 1-367, 1-320, and 1-316, respectively were cloned into the pGEX expression vector and transformed into *E. coli* (BL21) electro-competent cells. Protein expression was induced with 0.8 mM IPTG at 25°C for 3 hr and GST-tagged protein was purified using glutathione Sepharose beads (Sigma-Aldrich). Flag-tagged Tao-1 constructs for expression in S2 cells were gifts from S. Kobayashi's lab in Japan (Sato et al., 2007). Myc-tagged Hpo, V5/His-tagged Wts, and Myc-tagged Kibra were gifts from D.J. Pan's lab (Wu et al., 2003; Yu et al., 2010). Ex-HA and Mer-HA were gifts from G. Halder's lab (Hamaratoglu et al., 2006). Human TAOK3 cDNA was obtained from Thermo Scientific and subcloned into the pTFW destination vector of the Gateway System (Invitrogen) for expression in *Drosophila* S2 cells.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.devcel.2011.08.028.

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