A Screen for Conditional Growth Suppressor Genes Identifies the Drosophila Homolog of HD-PTP as a Regulator of the Oncoprotein Yorkie

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SUMMARY

Mammalian cancers depend on “multiple hits,” some of which promote growth and some of which block apoptosis. We screened for mutations that require a synergistic block in apoptosis to promote tissue overgrowth and identified myopic (mop), the Drosophila homolog of the candidate tumor-suppressor and endosomal regulator His-domain protein tyrosine phosphatase (HD-PTP). We find that Myopic regulates the Salvador/Warts/Hippo (SWH) tumor suppressor pathway: Myopic PPxY motifs bind conserved residues in the WW domains of the transcriptional coactivator Yorkie, and Myopic colocalizes with Yorkie at endosomes. Myopic controls Yorkie endosomal association and protein levels, ultimately influencing expression of some Yorkie target genes. However, the antiapoptotic gene diap1 is not affected, which may explain the conditional nature of the myopic growth phenotype. These data establish Myopic as a Yorkie regulator and implicate Myopic-dependent association of Yorkie with endosomal compartments as a regulatory step in nuclear outputs of the SWH pathway.

INTRODUCTION

The link between unrestrained proliferation and the evasion of apoptosis in vertebrate tumors is well established (e.g., Evan and Vousden, 2001). Many growth-promoting lesions such as amplification of c-Myc or the loss of Rb trigger compensatory apoptosis, which must then be overcome by antiapoptotic lesions in order for tumorigenesis to proceed. Many of the molecular mechanisms that drive tumorigenesis are conserved in Drosophila melanogaster, and in recent years, Drosophila has proven itself amenable to the study of cooperating mutations that drive tumor progression and metastasis (Brumby and Richardson, 2005; Chi et al., 2010; Pagliarini and Xu, 2003; Wu et al., 2010). In some cases, this cooperativity has been shown to arise from synergistic effects on cell proliferation and death pathways (Asano et al., 1996; Nicholson et al., 2009; Pellock et al., 2007; Staehling-Hampton et al., 1999), yet the extent to which compensatory apoptosis has hindered the identification of a conditional class of growth suppressor genes in Drosophila has not been comprehensively examined.

The conserved Salvador/Warts/Hippo (SWH) pathway controls a transcriptional program that includes both pro growth and antiapoptotic targets (Halder and Johnson, 2011; Pan, 2010). Pathway components include the protocadherin Fat, the apical membrane determinant Crumbs (Crb), and the FERM-domain proteins Expanded (Ex) and Merlin (Mer) (Bennett and Harvey, 2006; Chen et al., 2010; Cho et al., 2006; Grzeschik et al., 2010; Hamaratoglu et al., 2006; Ling et al., 2010; Robinson et al., 2010; Silva et al., 2006; Willecke et al., 2006). These factors regulate a core serine/threonine kinase cassette consisting of the Ste20-like kinase Hippo (Hpo) (Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003), which acts together with the scaffolding protein Salvador (Sav) (Kangos-Singh et al., 2002; Tapon et al., 2002) to phosphorylate the NDR family kinase Warts (Wts) (Justice et al., 1995; Xu et al., 1995). Wts then (Lai et al., 2005) phosphorylates the cotranscriptional activator Yorkie (Yki) on sites including Ser168 (S168) (Dong et al., 2007; Huang et al., 2005). This modification anchors Yki in the cytoplasm by recruiting 14-3-3 proteins (Oh and Irvine, 2008). In the absence of SWH signaling, Yki shuttles into the nucleus and, together with sequence-specific DNA binding factors (Goulev et al., 2008; Peng et al., 2009; Wu et al., 2008; Zhang et al., 2008; Zhao et al., 2008), activates a transcriptional program including the pro growth microRNA bantam, the pro differentiation gene cyclin E (cycE), the antiapoptotic gene diap1, and the upstream regulators ex and mer.

The SWH pathway has potent antigrowth activity (for review, see Pan, 2010; Zhao et al., 2010), and multiple mechanisms exist to limit Yki activity in developing tissues. While S168 is critical for Yki:14-3-3 binding, Wts phosphorylation of S111 and S250 also contribute to Yki inhibition (Oh and Irvine, 2009; Ren et al., 2010). Yki is directly inhibited in a phosphorylation-independent manner via interactions between two WW domains within Yki that are bound by PPxY sequence motifs in Hpo, Wts, and Ex (Badouel et al., 2009; Oh et al., 2009). Mammalian cells have additional regulatory mechanisms to control activity of the Yki homolog Yes-associated protein (YAP). YAP is tyrosine phosphorylated by c-Src/Yes kinases, which modulates the ability of YAP to recruit the Runx2 protein and control osteoblast differentiation (Zaidi et al., 2004). DNA damage triggers the c-Abl kinase to phosphorylate YAP on Y357, and this may bias YAP toward the promoters of apoptotic genes over growth-arrest genes (Levy et al., 2007; Staehling-Hampton et al., 1999). Yet the extent to which compensatory apoptosis has hindered the identification of a conditional class of growth suppressor genes in Drosophila has not been comprehensively examined.
Myopic Controls SWH Output by Binding to Yorkie

RESULTS

slaughterhouse-five Alleles Produce Conditional Tissue Overgrowth

We designed and implemented a screen for survival-dependent growth suppressor genes on Drosophila chromosome 3L. The screen used FLP recombination driven by the promoter of the eyeless (ey) gene (Newsome et al., 2000) to produce a mixture of mutant clones (unpigmented) and wild-type twin spots (pigmented red) in the adult eye (Figure 1A). To block cell death in mutant clones, mutagenesis was carried out using a parental FRT chromosome carrying the genomic deletion H99, which removes genes required for virtually all developmentally programmed cell death (White et al., 1994). H99 mutant clones display a block in developmental apoptosis in the pupal retina (30 hr after puparium formation [APF]) (see Figures S1A and S1A†) that exhibited a clonal growth advantage in the adult eye (Figures 1B versus 1D). We used this background to screen for recessive mutations that could synergize with H99 to confer a growth advantage and identified a recessive-lethal complementation group, which we named slaughterhouse-five (sfv), consisting of two alleles (F2.6.3 and F2.6.11) that exhibited a clonal growth advantage in the adult eye (Figure 1C) relative to an H99 control. Experiments were carried out with the sfv allele (F2.6.3) unless otherwise indicated. To test the survival-dependent nature of the sfv phenotype, the H99 deletion was removed. Adult sfv mosaic eyes are small and rough and contain little to no sfv mutant tissue (Figure 1E). Clones of sfv mutant cells in the larval eye disc contain elevated levels of cleaved caspase-3 (C3) (Figures S1B and S1B†), indicating they are normally eliminated by apoptosis. Thus the overgrowth of sfv mutant cells is conditional on a synergistic block in cell death provided by the H99 deletion.

sfv Is Allelic to myopic, the Drosophila Homolog of HD-PTP

The sfv lesions were mapped by deficiency mapping and candidate gene sequencing. This identified a nonsense mutation in the seventh exon of the gene CG9311 on the sfv chromosome. The
CG8311 gene corresponds to mop, which encodes the Drosophila homolog of vertebrate HD-PTP. The sfv\(^3\) mutation truncates Mop within its C-terminal region, just prior to the PTPase catalytic region (Figure S1H). The lesion in the sfv\(^{17}\) allele was not identified, but staining with an anti-Mop antibody shows reduced Mop protein levels in both sfv\(^3\)H99 and sfv\(^{17}\)H99 eye clones (Figures S1C and S1C; data not shown), indicating that both sfv alleles reduce Mop expression. The sfv\(^3\) and sfv\(^{17}\) alleles fail to complement existing mop alleles (data not shown) and will be referred to as mop\(^{sfv3}\) and mop\(^{sfv17}\).

Mop/HD-PTP is a conserved endosomal regulatory protein that contains an N-terminal Bro1 domain and a C-terminal predicted PTPase domain. The PTPase domain lacks activity in vitro due to an amino acid change in the phosphate-binding loop that diverges from all other active PTPases (Gingras et al., 2009), and this change is conserved in Mop. The Mop Bro1 domain is required to promote the endocytic trafficking and activity of the EGF and Toll receptors (Huang et al., 2010; Miura et al., 2008) and defects in differentiation of Elav-positive neurons within mop eye clones have been attributed to a defect in EGF signaling (Miura et al., 2008). However, some Elav-positive cells remain in mop\(^{sfv}\)H99 clones (Figures S1D and S1E), and the R2/R5 photoreceptor marker Rough is increased in mop\(^{sfv}\)H99 clones relative to mop\(^{sv}\) clones (Figures S1F and S1G). Thus, the lack of photoreceptors in mop mutant clones may be due to excess apoptosis and impaired EGF-dependent photoreceptor recruitment.

**mop Mutations Collaborate with a Block in Cell Death to Elicit Organ Overgrowth**

Genetic manipulations that simultaneously increase proliferation and reduce apoptosis often increase organ size. To test the effect of the mop\(^{sfv}\)H99 genotype on organ size, the recessive cell-lethal Minute technique was used to generate heads composed entirely of mop\(^{sfv}\)H99 cells (mop\(^{sfv}\)H99/M(3)). These animals die at the pharate adult stage with extra folds of head cuticle relative to control heads and eyes that are constricted at their margins and protrude from the head (Figures 2A–2D) and contain enlarged facets (data not shown). Generation of mop\(^{sfv}\)H99 clones throughout the body produced outgrowths in the adult thorax (Figures 2E and 2F) and increased haltere size (Figures 2G and 2H) relative to H99 controls. Thus, combined loss of Mop and the H99 proapoptotic genes increases the growth of multiple types of epithelia.

**mop Loss Increases Cell Proliferation Rates and Inhibits Cell-Cycle Exit**

The synergy between mop\(^{sv}\) and H99 alleles indicates that mop loss may increase cell number, conditional on a block in apoptosis. Staining with anti-Discs large (Dlg) to mark apical cell profiles reveals excess interommatidial cells (IOCs) in mop\(^{sfv}\)H99 pupal eye clones relative to H99 mutant clones (Figures 3A and 3B). Patterning defects are also evident within mop\(^{sfv}\)H99 mutant pupal clones, which may be a consequence of additional roles for Mop in cell fate pathways (e.g., Miura et al., 2008). Scattered ectopic S phase cells appear among mop\(^{sfv}\)H99 mutant cells posterior to the second mitotic wave (SMW) (Figures 3C–3F), which normally marks the point at which cells become postmitotic (Wolff and Ready, 1993); this pheno-

![Figure 2. mop Loss Cooperates with a Block in Death to Increase Organ Size](image)

(A and B) Bright-field images of control H99/M(3) (A) and mop\(^{sfv}\)/H99/M(3) (B) eyes/heads.

(C and D) Scanning electron micrographs (SEM) of control H99/M(3) (C) and mop\(^{sfv}\)/H99/M(3) (D) eyes/heads.

(E and F) SEM of H99 (E) and mop\(^{sfv}\)/H99 (F) clones in adult thoraces. mop\(^{sfv}\)/H99 mutant tissue is marked by dashes (\(\ldots\)).

(G and H) Images of mosaic H99 (G) and mop\(^{sfv}\)/H99 (H) halteres.

...type is not evident in H99 control clones (Figure 3D). mop\(^{sfv}\)/H99 mutant eye clones also show a perdurance of cyclin A and, to a lesser extent, cyclin E, posterior to the morphogenetic furrow (MF) (Figures 3G–3L). To test whether mop limits cell division, the rate of clonal expansion of H99 and mop\(^{sfv}\)/H99 clones were analyzed in the wing disc epithelium at fixed time points after clonal induction. mop\(^{sfv}\)/H99 clones are consistently larger and contain more cells than wild-type “twin spots” or control clones homozygous for the H99 deletion (Figures 3N and 3O); the boundaries of mop\(^{sfv}\)/H99 clones tend to be regular (Figure 3M, top left panel), suggesting that mop loss may affect cell adhesion. Fluorescence-activated cell sorting (FACS) analysis of mop\(^{sfv}\)/H99 and H99 wing disc cells relative to a control ubi > GFP chromosome indicates that mop\(^{sfv}\)/H99 cells have a DNA-content profile and size similar to H99 control cells (Figure 3M). Thus, although mop\(^{sfv}\)/H99 mutant cells proliferate more rapidly than control cells, this is not accompanied by an overall shift in cell-cycle phaseing.

**mop Promotes SWH Signaling and Functions Downstream of the Core SWH Kinase Cassette**

The mop\(^{sv}\)/H99 mutant growth phenotypes resemble those associated with Sav/Wts/Hpo (SWH) pathway mutants (for
Myopic Controls SWH Output by Binding to Yorkie

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Figure 3. mop Limits Cell Division

(A and B) Confocal images of H99 (A) and mop\textsuperscript{aps}H99 (B) pupal eye clones (40 hr APF) stained with anti-Dlg.

(C–L) Images of H99 (C and D) or mop\textsuperscript{aps}H99 (E–L) eye clones marked by the absence of GFP (green) and stained for BrdU incorporation (C–F), Cyclin A (G–I), or Cyclin E (J–L). The morphogenetic furrow (MF) is marked by an arrowhead in (C–L). Yellow arrows in (H) denote perdurance of Cyclin A (black line) early third instar wing discs.

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(N) or mop\textsuperscript{aps}H99 (O) clone: twin spot pairs. Data are arranged in order of increasing clone size. Variation in mop\textsuperscript{aps}H99 cell counts is due to position-specific effects in the wing.

(N and O) Cell number in individual H99 (N) or mop\textsuperscript{aps}H99 (O) clone: twin spot pairs. Data are arranged in order of increasing clone size. Variation in mop\textsuperscript{aps}H99 cell counts is due to position-specific effects in the wing.

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mirrors other peripheral regulators of the SWH pathway, which show mild IOC phenotypes when lost individually, but synergistic effects when combined (e.g., Baumgartner et al., 2010; Genevet et al., 2010; Ling et al., 2010; Yu et al., 2010).

Mop Interacts with Yki via Residues in the Yki WW Domains

To identify the protein target of Mop within the SWH pathway, we undertook an affinity purification/mass spectrometry (AP/MS) analysis of Mop-containing complexes (Kyriakakis et al., 2008; Veraksa et al., 2005) purified from cultured S2 cells. A form of Mop with a point mutation in the putative PTPase catalytic domain (C1728 to alanine; MopCS) was used for this analysis in order to enhance interactions with endogenous proteins. This technique identified 14 partially overlapping peptides derived from the endogenous Yki protein (bolded in Figure 5A). Coimmunoprecipitation (coIP) analysis from S2 cells expressing HA-Yki and Mop-V5 confirmed the interaction in the reciprocal orientation: HA-Yki is able to efficiently coIP both MopCS and wild-type Mop (MopWT) (Figures 5B and 5C). To study the Mop:Yki interaction further, two conserved tyrosines (Y281 and Y350) within the Yki WW domains (see Figure 5A) were individually altered to alanine (HA-YkiY281A and HA-YkiY350A). Although expressed at similar levels to HA-Yki, both of the Y-to-A WW mutants are defective in binding to MopWT and MopCS (Figures 5B and 5C). Mop contains two candidate PPxY motifs located in its linker region between the Bro1 domain and the PTPase-like domain (Figure 5D), indicating that Mop may bind Yki via a WW:PPxY interaction module similar to the interaction between the Yki WW domains and PPxY motifs in Ex, Hpo, and Wts (Badouel et al., 2009; Oh et al., 2009). To test this, each of the Mop PPxY motifs were changed to PPxA.
(PPxY1A, PPxY2A, or PPxY1,2A) and tested for Yki binding. The Mop-PPxY1,2A mutant showed a loss of Yki binding, while each single mutant showed minor residual binding (Figure 5D and data not shown). Thus, Mop can bind to Yki via a WW:PPxY interaction mechanism that is conserved in other SWH proteins that bind directly to Yki.

Mop Inhibition of Yki Is Phosphorylation Independent

A version of Yki carrying serine-to-alanine mutations in three Wts phosphorylation sites (S111A, S168A, and S250A; UAS-yki:V5S3A) shows enhanced oncogenic activity due to a loss of inhibitory phosphorylation by Wts (Oh and Irvine, 2009). However, expression of Hpo, Wts, or Ex, which directly bind the Yki WW domains via their PPxY motifs, are able to suppress YkiS3A phenotypes (Oh et al., 2009). To test whether Mop can also regulate Yki in a phosphorylation-independent manner, UAS-yki:V5 or UAS-yki:V5S3A was coexpressed with Mop (UAS-mop) in the larval eye. As reported, Yki:V5 shows little effect on growth of the larval eye disc while Yki:V5S3A produces enlarged disc with folds of excess tissue (Figures 5E and 5F). Expression of Mop resulted in substantial suppression of the Yki:V5S3A growth phenotype (Figure 5G). This ability of Mop to antagonize a form of Yki that is refractory to SWH-mediated phosphorylation is consistent with a model in which Mop inhibits Yki directly via the WW:PPxY interaction. Significantly, heterozygosity for mop enhances the progrowth effect of the human Yki homolog Yap in the developing eye (Figures 5H–5J), suggesting that the functional relationship between Yki and Mop-like proteins may be conserved.

mop Regulates Association of Yki with Endosomal Compartments

Studies with HD-PTP indicate that the Mop N-terminal Bro1 domain may facilitate interactions with ESCRT endosomal complexes (Doyotte et al., 2008; Ichioka et al., 2007; Odorizzi et al., 2003) and that this underlies the ability of Mop to promote signaling by the EGF and Toll receptors (Huang et al., 2010; Miura et al., 2008). The finding that Mop sequences outside the Bro1 domain bind Yki suggests that Mop may regulate the association of Yki with specific endosome-associated protein complexes, or that Mop physically links Yki to the endosomal trafficking of a receptor-like SWH component such as Fat (for review, see Reddy and Irvine, 2008). To test whether the mopH99 growth phenotype requires signaling downstream of Fat, we expressed an RNAi knockdown transgene to approximate (app), which is required for the overgrowth of mutant fat tissue (Matakatsu and Blair, 2008), in the mop/M(3)
background. Knockdown of app had no effect on the size of mopsfvH99/M(3) heads, and reciprocally, loss of Mop had no discernible effect on Fat levels or the degree of Fat colocalization with the early endosomal marker EEA1 (Figures S2E–S2H), indicating that mopsfvH99 may promote growth independent of a defect in Fat signaling.

The effect of mopIR on Yki protein (see Figures S2I and S2J) led us to examine the relationship between Mop and the cytoplasmic pool of Yki more closely. In imaginal discs and S2 cells, Mop is normally found in cytoplasmic puncta adjacent to Rab5-positive endosomes and partially colocalizes with Rab7-positive endosomes (Huang et al., 2010; Miura et al., 2008). To test whether the physical interaction of Mop and Yki proteins is accompanied by a colocalization of the proteins to specific cytoplasmic structures, HA–Yki and Mop–V5 were visualized in cultured S2 cells. HA–Yki and Mop–V5 substantially colocalize to discrete puncta in the cytoplasm (Figures 6A and 6A′). To identify these Yki-containing structures, Yki:V5 was coexpressed with either GFP:Rab7 or GFP:Rab5 to mark late and early endosomes, respectively. A majority of Yki:V5 protein localizes to Rab7-containing late endosomes in S2 cells (Figures 6B–B′). This parallels the reported partial location of Mop to Rab7 endosomes (Miura et al., 2008) and indicates that Mop and Yki partially colocalize at these structures. By contrast, Yki:V5 is largely excluded from Rab5-containing early endosomes (Figures 6C and 6C′).

As reported (Miura et al., 2008), depletion of mop by RNA interference caused an enlargement of GFP:Rab7 endosomes (Figures 6E and 6E′). These enlarged mop-knockdown GFP:Rab7 endosomes are depleted for Yki:V5 relative to untreated S2 cells (green arrowheads, Figures 6E and 6E′), and in parallel, Yki:V5 appears in GFP:Rab5 endosomes (yellow arrowheads, Figures 6D and 6D′). This endosomal redistribution of exogenous Yki correlates with an increase in overall levels of the protein and increased expression of the Yki target ex as measured by qRT-PCR (Figures S3A and S3C). A similar rise in endogenous Yki levels is observed upon mop knockdown in S2 cells.
cells, and this correlates with elevated expression of ex (Figures S3B and S3C). Moreover, when expressed together with HA-Yki, Mop can retard Yki-driven transcriptional upregulation of ex (Figure S3C).

We next examined whether mop loss in imaginal disc tissue affects endosomal distribution of endogenous Yki. In control H99 clones, Yki localizes to punctate cytoplasmic structures that occasionally colocalize with early endosomal antigen-1 (EEA1)-positive early endosomes (Figures 6F–6H). In mopΔH99 mutant tissue, EEA1-positive endosomes are more abundant and tend to be larger than in control cells (red arrows in Figures 6H and 6K). Yki also accumulates in punctate structures in mopΔH99 that substantially overlap with enlarged EEA1-positive endosomes (Figures 6I–6K). The extent of Yki:EEA1 colocalization was quantitated by counting the percentage of EEA1-positive endosomes of all sizes that are also positive for Yki. In H99 control clones, the percentage of EEA1/Yki-positive endosomes is 19% ± 2% (compiled from ten clones containing a total of 380 EEA1-positive endosomes). In mopΔH99 tissue this approximately doubles to 38% ± 3% (compiled from ten clones containing a total of 584 EEA1-positive endosomes). To determine whether this enrichment of Yki in EEA1 endosomes is accompanied by changes in localization of Yki on late endosomes, the association of Yki with Rab7-positive late endosomes was examined in H99 and mopΔH99 clones. In H99 control clones, Yki localizes to structures that are adjacent to but do not substantially overlap with Rab7 late endosomes (Figures S4E and S4E′). mopΔH99 clones contain enlarged Rab7-positive endosomes that exclude the increased pool of cytoplasmic endogenous Yki (Figures S4D and S4D′). Thus, mop loss in imaginal disc tissue leads to an increase in the early-endosome-associated pool of cytoplasmic Yki.

**Mop Restricts Ectopic ex but Not diap1 Transcription in Imaginal Discs**

Yki lacks DNA binding activity but has been shown to activate transcription of the diap1 promoter via the DNA binding factor and TEAD homolog Scalloped (Sd) (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008). Interestingly, qRT-PCR analysis shows that mopΔH99 mutant discs elevated expression of ex mRNA transcripts but not diap1 transcripts, while wts mutant cells elevated levels of both ex and diap1 transcripts (Figure 7B). In addition, DIAP1 protein is not elevated in mopΔH99 mutant clones (Figures 7A and 7A′) but is readily detected at elevated levels in wts mutant eye disc clones (Figures S4A and S4A′). diap1-lacZ (th-Z) reporter expression is also unaffected in mopΔH99 mutant eye clones marked by upregulated Crb (Figures S4B and S4B′). The lack of an effect on diap1 indicates that Mop may be required to regulate the expression of some SWH targets and not others. To test this, MARCM analysis was used to analyze SWH target gene induction in wts mutant clones overexpressing Mop. As previously reported (Hamaratoglu et al., 2006), ex-Z and th-Z are strongly upregulated in wts mutant clones (Figures 7C, 7C′, 7D, and 7D′). Overexpression of wild-type Mop rescues the effect of wts loss on ex-Z (Figures 7E and 7E′) but does not block elevated th-Z expression (Figures 7F and 7F′). The catalytic site mutant form of MopCS (Miura et al., 2008) was also able to efficiently suppress ectopic ex-Z levels in wts clones (Figures 7G and 7G′). These data suggest that Mop acts to restrict the enrichment of Yki in early endosomes, but not diap1 transcription and that it does so through a mechanism that does not require Mop catalytic tyrosine phosphatase activity.

**DISCUSSION**

Here, we describe a screening strategy to identify mutations in *Drosophila* that require a synergistic block in cell death in order
to promote tissue overgrowth. Using this approach, we have identified the endosomal protein Mop, which is the Drosophila homolog of the candidate mammalian tumor suppressor HD-PTP, as a regulator of the SWH growth inhibitory pathway. Through multiple approaches, we demonstrate that Mop regulates Yki activity via a mechanism involving direct binding and modulation of Yki endosomal association.

This study defines a pool of cytoplasmic Yki that binds Mop and colocalizes with it on endosomes. Data from discs and cultured cells indicate Mop controls endosomal association of this pool of Yki and that a positive correlation exists between Yki colocalization with EEA1-positive early endosomes and Yki levels and activity. A growing body of genetic and molecular data support a role for endosomes as key signaling centers for signal transduction pathways that influence the nuclear translocation of latent cytoplasmic transcription factors (Birtwistle and Kholodenko, 2009; Bokel et al., 2006; Devergne et al., 2007; Di Guglielmo et al., 2003; Fortini and Bilder, 2009) (for review, see Miaczynska et al., 2004; Murphy et al., 2009; Taelman et al., 2010). For example, the activated c-Met receptor associates with the STAT3 transcription factor on EEA1-positive endosomes prior to STAT3 nuclear accumulation, and c-Met delivery to a perinuclear endosomal compartment is necessary to sustain nuclear STAT3 (Kermorgant and Parker, 2008). The enrichment of Yki on EEA1 endosomes and activation of a subset of Yki nuclear targets in mop mutant cells suggests that Yki, perhaps in association with receptor complexes, may take a similar route to the nucleus. Intriguingly, microtubule-regulated perinuclear transport of Merlin (Mer) controls nucleocytoplasmic shuttling of Yki (Bensenor et al., 2010). The direct link between Mer transport and Yki shuttling is not clear. However, as Mer can control internalization of transmembrane receptors (for review, see McClatchey and Felchon, 2009), perinuclear transport of Mer might in turn modulate endosomal internalization and transit of Yki:receptor complexes en route to the nucleus.

Genetic data show that exogenous Mop is sufficient to restrict ectopic expression of the Yki-target ex but not diap1 and that loss of endogenous Mop upregulates a set of Yki targets that do not include diap1. Mop thus appears to define a regulatory step in determining outputs of the SWH pathway, perhaps as part of the endosomal sorting process. Trafficking of transmembrane proteins down alternate endosomal routes contributes to the activation of different nuclear programs in the Notch, Jak/STAT, and Akt pathways (Hori et al., 2004; Kermorgant and Parker, 2008; Schenck et al., 2008). Similarly, association of Yki-containing complexes with different endosomal compartments may shift Yki nuclear output, perhaps by bringing Yki into contact with posttranslational modifiers or binding partners that affect its ability to activate its site of target promoters. Further studies will be required to establish whether loss of Mop indeed alters Yki posttranslational modification or the assembly of Yki transcriptional complexes.

In the context of SWH signaling, the differential effect of mop loss on ex and diap1 expression place Mop within the growth regulatory arm of the SWH network. Differential effects on the growth and apoptotic outputs of the SWH pathway is also a feature of mutations in ex and mer, which preferentially drive Yki-dependent clonal growth or antiapoptotic signals, respectively (Pellock et al., 2007) and whose combined mutant pheno-
mechanism of growth suppression by HD-PTP is not known, but its ability to suppress colony formation of human renal cancer cells is independent of catalytic PTPase activity (Gingras et al., 2003) in much the same way that regulation of Yki by Mop does not require PTPase activity. Although HD-PTP lacks a canonical PpXY motif, genetic data indicate that Mop retains the ability to inhibit Yap activity in the Drosophila eye. The extent to which HD-PTP binds Yap or Tau has yet to be examined, but if the relationship between the orthologous Drosophila proteins is conserved in vertebrates, this link to Yki/Yap may contribute to growth regulatory roles of vertebrate HD-PTP proteins in development and disease.

**EXPERIMENTAL PROCEDURES**

**Drosophila Genetics**

Alleges used: exD8(1:10) (Boedigheimer et al., 1993), thD169 (th-Z) (Hay et al., 1995), ykiD10 (Huang et al., 2005), wtsC1 (Xu et al., 1995), and H99 (White et al., 1994). Transgenes used: UAS-Myo-Wts1 (Jia et al., 2003), UAS-Ex (Boedigheimer et al., 1997), UAS-Mop and UAS-MopC10 (Miura et al., 2008), UAS-Yki:V5 (Oh and Irvine, 2009), UAS-mopC10 (Vienna Drosophila RNAi Collection), and bantam-GFP (Brennecke et al., 2003).

**Immunofluorescence**

Imaginal discs and S2 cells were fixed and stained following standard procedures. Antibodies used: mouse anti-Dlg (1:20, DSHB), mouse anti-BrdU (1:50, Becton Dickinson), mouse anti-cyclin E (1:5, H. Richardson), mouse anti-cyclin B (1:800, DSHB), and guinea pig anti-Expanded (1:10,000, R. Fehon), rat anti-Crb (1:500, C. Bergmann), mouse anti-Repo (1:50, R. Graveley), mouse anti-c-Jun (1:100, U. Tepass), mouse anti-Wg (1:50, DSHB), and mouse anti-A (1:200, DSHB), guinea pig anti-Expanded (1:10,000, R. Fehon), rat anti-Crb (1:500, U. Tepass), mouse anti-Wg (1:800, DSHB), mouse anti-β-galactosidase (1:1000, Promega), mouse anti-V5 (1:200, Invitrogen) and mouse anti-HA (1:100, Sigma). Proteins were induced with 0.35 mM CuSO4 overnight. To establish a Roche LightCycler 480. All reactions were performed in triplicate, and the relative amount of diap1 and ex mRNA was normalized to β-tubulin transcript.

**Clonal Cell Counts and Cell-Cycle Analysis**

For FACS analysis, H99/M(3) and mopC10/Mop/M(3) wing discs were dissociated in PBS Trypsin-EDTA and stained with 20 µM DRAQ-5 (Biostatus Limited). Data were acquired on a Becton Dickinson LSRII flow cytometer and analyzed with FlowJo Software. Clonal cell count data were generated by producing heat-shocked H99 and mopC10 wing clones 48 hr AED. At 96 AED, wing discs were fixed and stained with DRAQ5, and the number of nuclei per clone and twin-spot pair was counted.

**Quantitative RT-PCR**

Total RNA was extracted from larval eye discs using TRIzol (Invitrogen) and purified using RNeasy Mini Kit (QIAGEN). Superscript II RT and random primers (Invitrogen) were used to produce cDNAs. Exon-specific primers were used with SYBR Green I Master (Roche) to perform qPCR reactions using a Roche LightCycler 480. All reactions were triplicated, and the relative amount of diap1 and ex mRNA was normalized to β-tubulin transcript.

**Cell Culture**

Drosophila S2 cells were maintained at 25°C in Schneider’s Drosophila medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO). Cells were incubated with 0.35 mM CuSO4 overnight. To establish a stable cell line of pmK33-GSNATP-MopC10, S2 cells were transfected using Effectene transfection reagent (QIAGEN). After 48 hr of incubation with the transfection reagent, cells were maintained in complete media with 300 µg/ml hygromycin (Sigma). mop dsRNA was generated using the T7 Ribopmax system (Promega) using the following primers: 5’- T7-T6GGCCACATTCGAGATATCG-3’ and 5’-T7-TTCGCGATGTTTGCTGAG-3’. mop dsRNA was transfected into cells using Cellfectin (Invitrogen), and cells were incubated for 48 hr, followed by transfection with the following constructs: (1) pAc5.1-Yki:V5, (2) UAS-GFP:Rab5, (3) UAS-GFP:Rab7, (4) pM-TG4, followed by another 48 hr incubation and induction with 0.5 mM CuSO4 for 4 hr.

**Immunoprecipitation**

Cell extracts were lysed in lysis buffer (LB: 50 mM Tris [pH 7.5], 125 mM NaCl, 5% glycerol, 0.2% IGEpal, 1.5 mM MgCl2, 1 mM DTT, 25 mM NaF, 1 mM Na2VO4, 1 mM EDTA, and complete protease inhibitor [Roche]), and lysates were incubated with anti-HA affinity beads (Sigma) for 2 hr at 4°C, followed by extensive washes. Protein complexes were eluted with SDS sample buffer, separated on SDS protein gels, transferred onto Immun-Blot PVDF membranes (Bio-Rad), and probed with mouse anti-HA or mouse anti-VS antibodies (Sigma).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, and four figures and can be found with this article online at doi:10.1016/j.devcel.2011.04.012.

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**REFERENCES**


