Differential requirement of Salvador-Warts-Hippo pathway members for organ size control in *Drosophila melanogaster*

Claire C. Milton, Xiaomeng Zhang, Nathaniel O. Albanese and Kieran F. Harvey*

**SUMMARY**

The Salvador-Warts-Hippo (SWH) pathway contains multiple growth-inhibitory proteins that control organ size during development by limiting activity of the Yorkie oncoprotein. Increasing evidence indicates that these growth inhibitors act in a complex network upstream of Yorkie. This complexity is emphasised by the distinct phenotypes of tissue lacking different SWH pathway genes. For example, eye tissue lacking the core SWH pathway components *salvador*, *warts* or *hippo* is highly overgrown and resistant to developmental apoptosis, whereas tissue lacking *fat* or *expanded* is not. Here we explore the relative contribution of SWH pathway proteins to organ size control by determining their temporal activity profile throughout *Drosophila melanogaster* eye development. We show that eye tissue lacking *fat*, *expanded* or *discs overgrown* displays elevated Yorkie activity during the larval growth phase of development, but not in the pupal eye when apoptosis ensues. Fat and Expanded do possess Yorkie-repressive activity in the pupal eye, but loss of *fat* or *expanded* at this stage of development can be compensated for by Merlin. Fat appears to repress Yorkie independently of Dachs in the pupal eye, which would contrast with the mode of action of Fat during larval development. Fat is more likely to restrict Yorkie activity in the pupal eye together with Expanded, given that pupal eye tissue lacking both these genes resembles that of tissue lacking either gene. This study highlights the complexity employed by different SWH pathway proteins to control organ size at different stages of development.

**KEY WORDS:** *Drosophila*, Hippo pathway, Organ size control

**INTRODUCTION**

The Salvador-Warts-Hippo (SWH) pathway is a crucial determinant of organ size in both *D. melanogaster* and mammals (reviewed by Harvey and Tapon, 2007; Pan, 2007; Reddy and Irvine, 2008). Altered regulation of this pathway has also been implicated in the genesis of human tumours (reviewed by Harvey and Tapon, 2007; Pan, 2007; Reddy and Irvine, 2008). The SWH pathway controls the size of imaginal discs (the presumptive adult *D. melanogaster* organs) by regulating cellular growth rates and cell proliferation. The SWH pathway also controls organ size by modulating apoptosis, at least in the *D. melanogaster* eye. The adaptor protein Salvador (Sav), and the Ser/Thr kinase Warts (Wts), were the first proteins recognised to function in this pathway (Tapon et al., 2002). Since then, at least ten additional proteins have been implicated as SWH pathway members, including the Hippo (Hpo) Ser/Thr kinase, Mob as tumor suppressor (Mats), the Yorkie (Yki) transcriptional enhancer, the Band 4.1 proteins Expanded (Ex) and Merlin (Mer), the atypical cadherins Fat (Ft) and Dachsous (Ds), the Discs overgrown (Dco) Ser/Thr kinase, Dachs (D), Ras association family member (Rassl) and the Scalloped (Sd) transcription factor (Bennett and Harvey, 2006; Cho et al., 2006; Goulev et al., 2008; Hamaratoglu et al., 2006; Harvey et al., 2003; Huang et al., 2005; Jia et al., 2003; Kango-Singh et al., 2002; Lai et al., 2005; Polesello et al., 2006; Rogulja et al., 2008; Silva et al., 2006; Tapon et al., 2002; Tyler and Baker, 2007; Udan et al., 2003; Willecke et al., 2006; Willecke et al., 2008; Wu et al., 2003; Wu et al., 2008; Zhang et al., 2008). The SWH pathway controls organ size at least in part by regulating the transcription of key downstream target genes including *Cyclin E* (*CycE*), which drives the transition from G1 to S phase, *Drosophila inhibitor of apoptosis 1* (DIAP1; thread – FlyBase), the *bantam* (*ban*) microRNA and *ex* (Hamaratoglu et al., 2006; Nolo et al., 2006; Tapon et al., 2002; Thompson and Cohen, 2006; Udan et al., 2003; Wu et al., 2003).

Tissues that lack genes that promote SWH pathway activity share several phenotypic characteristics (Harvey and Tapon, 2007; Pan, 2007; Reddy and Irvine, 2008). These include increased rates of cell growth and proliferation in imaginal disc tissues with asynchronously dividing cells, ectopic proliferation posterior to the morphogenetic furrow in the *D. melanogaster* third instar larval eye, and increased expression of *CycE*, DIAP1 and *ex* in third instar larval imaginal discs. Observation of these common phenotypic traits has facilitated the classification of new SWH pathway members. Importantly, tissue that is deficient for individual SWH pathway proteins also exhibits phenotypic differences, suggesting that not all members of this pathway participate in the control of the same biological processes. For example, *ft* and *ex* are required to limit cell growth and proliferation via the SWH pathway but, unlike *sav*, *wts* and *hpo*, they are dispensable for the majority of apoptosis that occurs at the mid-pupal phase of development in the *D. melanogaster* eye (Bennett and Harvey, 2006; Pellock et al., 2006; Silva et al., 2006). This prompted us to classify components of the SWH pathway that have distinct roles in controlling cell growth, proliferation and apoptosis (such as *sav*, *wts*, *hpo* and *yki*) as core pathway members, and proteins that control some, but not all, of these processes as non-core pathway members (such as *ft* and *ex*), although this classification has warrant further investigation (Harvey and Tapon, 2007).

Here we show that tissue that lacks *ft*, *ex* or *dco* displays elevated Yki activity during larval, but not pupal, development. Ft and Ex do possess Yki-repressive activity in the pupal eye, but...
loss of these proteins can be compensated for by Mer. Interestingly, Ft appears to repress Yki independently of Dachs in the pupal eye, which contrasts with its mode of action during larval imaginal disc development. Ft is likely to restrict Yki in an Ex-dependent fashion in the pupal eye given that Yki activity in ft,ex double-mutant pupal eye tissue closely resembles that of ft or ex tissue.

MATERIALS AND METHODS

D. melanogaster stocks

Loss-of-function tissue harbouring mutations in different genes was generated by producing transheterozygous animals, or in a clonal fashion using the Flp/FRT technique, and the following mutant alleles: hpo<sup>+/+/et</sup> (Wu et al., 2003), ft<sup>+/et</sup> (Bryant et al., 1988), ft<sup>+/et2</sup> (Rawls et al., 2002), ex<sup>+/et</sup> (Pellock et al., 2006), ex<sup>+/et</sup> (Boedigheimer and Laughon, 1993), dco<sup>+/et</sup> (Zilian et al., 1999), Mer<sup>+/et</sup> (Fehon et al., 1997). Double-mutant clones were produced using Flp<sup>+/M40</sup> FRT40A (generated by meiotic recombination), or y<sup>exR</sup>, a FRT28 yki rescue construct (Huang et al., 2005) and either wts<sup>+/et</sup> (Xu et al., 1995) or dco<sup>+/et</sup>. To generate double-mutant clones containing Mer, the Mer rescue construct stock P[w<sup>+/et</sup> y w Mer4 FRT19A/Y; P[w<sup>+/et</sup> FRT19A]; +; eyFlp/TM6B was used (Hamaratoglu et al., 2006; McCartney et al., 2000). Double-mutant clones for Mer:ex were generated using y w Mer<sup>+/et</sup> FRT19A/Y; P[w<sup>+/et</sup> Mer4] FRT40A/ex<sup>et</sup> FRT40A; hsFLP MKRS<sup>+/et</sup> or y w Mer<sup>+/et</sup> FRT19A/Y; P[w<sup>+/et</sup> Mer4] FRT40A/ex<sup>et</sup> FRT40A; hsFLP MKRS<sup>+/et</sup>. Similarly, double-mutant clones for Mer:ft were generated using y w Mer<sup>+/et</sup> FRT19A/Y; P[w<sup>+/et</sup> Mer4] FRT40A/ex<sup>et</sup> FRT40A; hsFLP MKRS<sup>+/et</sup>. Triple-mutant clones for Mer:ft:dachs were generated using y w Mer<sup>+/et</sup> FRT19A/Y; P[w<sup>+/et</sup> Mer4] FRT40A/ex<sup>et</sup> FRT40A; hsFLP MKRS<sup>+/et</sup>. Other stocks included th<sup>58</sup> (Hay et al., 1995), ey<sup>297</sup> (Boedigheimer and Laughon, 1993), bantam-GFP sensor transgene (Brennecke et al., 2003), dco<sup>+/et</sup> (Zilian et al., 1999), y<sup>exR</sup> (Huang et al., 2005), UAS-ex RNAi (Dietzl et al., 2007), th<sup>18-4Gal4</sup>, y ey:Flp; FRT40A W[w<sup>+/et</sup> + ubi-GFP], y w hsFlp; FRT40A P[w<sup>+/et</sup> ubi-GFP], y w hsFlp; FRT40A P[w<sup>+/et</sup> arm-lacZ], y w eyFlp; FRT42D P[w<sup>+/et</sup> ubi-GFP], y w eyFlp; FRT82B P[w<sup>+/et</sup> ubi-GFP], y w hsFlp; FRT82B P[w<sup>+/et</sup> arm-lacZ], y w P[w<sup>+/et</sup> ubi-GFP] FRT19A and eyFlp, w P[mini W+w arm-lacZ] FRT19A +; eyFlp/TM6B.

Immunohistochemistry

Primary antibodies used were directed against DIAPI (from B. Hay) (Yoo et al., 2002), Ex (from A. Laughon) (Boedigheimer and Laughon, 1993), Ft (this study), Dic (from J. Price) (Muskus et al., 2007), Mer (McCartney and Fehon, 1996), Dlg and Actin (Developmental Studies Hybridoma Bank) and probed with anti-Ex (Boedigheimer and Laughon, 1993), anti-Dico (Muskus et al., 2007) or anti-Actin (clone JLA20, Developmental Studies Hybridoma Bank) as described (Zhang et al., 2009). Secondary antibodies were anti-rabbit-HRP and anti-mouse-HRP (Jackson Immunochemicals).

RESULTS

Core SWH pathway members control pathway activity throughout larval and pupal eye development

We and others recently discovered that the cadherin Ft controls tissue growth by signalling to downstream components of the SWH pathway (Bennett and Harvey, 2006; Cho et al., 2006; Silva et al., 2006; Tyler and Baker, 2007; Willecke et al., 2006). Interestingly, we found that although Ft controlled cell growth, proliferation and expression of SWH pathway target genes during larval stages of development, it was dispensable for the developmental apoptosis that occurs in the D. melanogaster eye at the mid-pupal phase (Bennett and Harvey, 2006). Similar results were described with regard to the function of the Ex protein (Bennett and Harvey, 2006; Hamaratoglu et al., 2006; Pellock et al., 2006). This is in contrast to core SWH pathway components such as Hpo, Sav, Wts and Yki, which control cell growth and proliferation during the larval phase of development, as well as apoptosis during pupal development (Harvey et al., 2003; Huang et al., 2005; Jia et al., 2003; Kango-Singh et al., 2002; Pantalacci et al., 2003; Tapon et al., 2002; Udan et al., 2003; Wu et al., 2003). The reason for this discrepancy was unclear and was especially surprising given that the key anti-apoptotic protein DIAPI was found to accumulate in tissue lacking either ft or ex (Bennett and Harvey, 2006; Cho et al., 2006; Pellock et al., 2006; Silva et al., 2006; Tyler and Baker, 2007; Willecke et al., 2006). A caveat to this result was that DIAPI expression was monitored at the third instar larval stage of development, when organs are still growing, and not at the mid-pupal stage of development, when apoptosis removes supernumerary cells in the D. melanogaster eye. Therefore, we hypothesized that Ft and Ex control SWH pathway activity in the D. melanogaster eye in a temporarily restricted fashion, i.e., during larval, but not pupal, development.

To investigate this hypothesis further, we analysed SWH pathway activity in eye tissue lacking core or non-core SWH pathway genes by measuring the expression of the well-characterised SWH pathway target genes DIAPI, ex and ban. Expression levels were determined at both the third instar larval and mid-pupal [28 hours after puparium formation (APF)] stages of development. Antibodies and gene-enhancer trap lines were used to record expression of DIAPI and Ex, whereas a green fluorescent protein (GFP) sensor was used to report expression of the ban microRNA (Brennecke et al., 2003). In all of our clonal analyses, wild-type and heterozygous tissue were marked by the core SWH pathway component hpo, we observed increased expression of DIAPI mRNA (Fig. 1A-B) and protein (Fig. 1G-H), and of Ex mRNA (Fig. 1C-D) and protein (Fig. 1I-J). We also observed elevated ban expression in hpo clones, evident as a reduction in intensity of the ban sensor, at both the larval and pupal stages of eye development (Fig. 1E-F'). Reduction of ban sensor was not uniform in pupal hpo eye clones, but levels were much lower than in wild-type tissue. Similar results were observed for expression of DIAPI, Ex and ban in tissue lacking either sav or wts (data not shown). These data are consistent with previous observations that core SWH pathway components control pathway activity throughout larval and pupal eye development of D. melanogaster (Harvey et al., 2003; Huang et al., 2005; Jia et al., 2003; Kango-Singh et al., 2002; Pantalacci et al., 2003; Tapon et al., 2002; Udan et al., 2003; Wu et al., 2003).
were detected using antibodies specific for these proteins. As reported previously, larval eye tissue lacking hpo42-47 APF (B,D,F,H,J) mosaic for expression is evident as a reduction in expression of the expression is reported using an antibody specific for this protein (red in E-F). Expression of DIAP1 (red in G-H) and Expanded (red in I-J) were detected using antibodies specific for these proteins. hpo42-47 mutant clones lack expression of GFP (green in A'-D',G'-J') or lacZ (green in E',F') and are merged with gene or protein expression images in A'-J'. Scale bars: 20 μm.

Eye tissue lacking ft or ex displays elevated Yki activity during larval, but not pupal, development.

To address whether the non-core SWH pathway proteins Ft and Ex control pathway activity in a temporally restricted fashion, we performed similar analyses to those in Fig. 1 on ft and ex mosaic eye tissues. As reported previously, larval eye tissue lacking ex displayed substantial elevation of DIAP1 mRNA (Fig. 2A), DIAP1 protein (see Fig. S1A in the supplementary material) and Ex protein (Fig. 2C) (Hamaratoglu et al., 2006; Pellock et al., 2006; Silva et al., 2006; Tyler and Baker, 2007). Expression of ban was slightly elevated in larval ex clones, as evidenced by a partial reduction in the ban sensor (Fig. 2E). Similar results were observed for DIAP1 mRNA, DIAP1 protein and ban expression in larval eye tissue lacking ft (Fig. 2G,I and see Fig. S1C in the supplementary material). We did not measure Ex protein expression in ft tissue as we and others have previously shown that whereas ex mRNA is elevated in ft eye tissue, total Ex protein levels are unchanged (Bennett and Harvey, 2006; Silva et al., 2006; Willecke et al., 2006). In pupal tissue lacking ex, DIAP1 mRNA (Fig. 2B), DIAP1 protein (see Fig. S1B in the supplementary material), Ex protein (Fig. 2D) and ban (Fig. 2F) levels were not elevated when compared with neighbouring wild-type tissue. Similarly, in pupal eye tissue lacking ft, DIAP1 mRNA (Fig. 2H), DIAP1 protein (see Fig. S1D in the supplementary material) and ban expression (Fig. 2J) were not elevated. DIAP1 expression was also unchanged in ft, ex double-mutant pupal eye clones, but was elevated in larval clones of this genotype (Fig. 2K-L'). This expression analysis suggests that Ft and Ex control SWH pathway activity during larval, but not pupal, eye development, consistent with the previous observation that Ft and Ex are dispensable for pupal retinal apoptosis (Bennett and Harvey, 2006; Silva et al., 2006; Tyler and Baker, 2007).
Recessive dco\(^2\) eye tissue displays elevated Yki activity during larval, but not pupal, development

Previously, recessive dco\(^1\) tissue was shown to phenocopy several traits associated with tissue deficient in tumour suppressor genes of the SWH pathway; these traits included overgrown imaginal discs and increased expression of Cyclin E and DIAP1 in third instar larval imaginal discs (Cho et al., 2006). dco\(^2\) was also shown to influence the stability of the Wts protein, suggesting that Dco is a SWH pathway component (Cho et al., 2006). To further explore a potential role for Dco in regulation of the SWH pathway, we performed dominant modification and genetic epistasis experiments. We found that Yki was rate-limiting for overgrowth of tissues caused by dco mutations, either in a clonal (dco\(^2\)) or transheterozygous (dco\(^2/dco_3\)) setting, providing further evidence that Dco controls tissue growth via the SWH pathway (see Fig. S2 in the supplementary material).

Recently, mechanistic insight was gained into Dco-mediated regulation of SWH pathway activity with the discovery that Dco influences phosphorylation of the Ft cytoplasmic domain and that the dco\(^2\) allele encodes a dominant-negative protein that blocks Fat phosphorylation (Badoel et al., 2009; Feng and Irvine, 2009).

In order to investigate whether Dco functions similar to core, or non-core, SWH pathway proteins, we investigated the phenotype of dco\(^2\) mutant tissue further. Initially, we examined the phenotype of dco\(^1\) mosaic eyes generated by the eyFlip system. dco\(^2\) tissue (white) was over-represented compared with control tissue (red or orange) suggesting that it has a relative growth advantage (Fig. 3A). In addition, the head cuticle was convoluted and overgrown, phenotypes reminiscent of eyes mosaic for either ft or ex (Fig. 3B), but distinct from eyes lacking core SWH pathway genes such as sav, wts or hpo, which are vastly overgrown and highly convoluted (Fig. 3C). The larger size of eyes lacking sav, wts or hpo is probably due to defective developmental apoptosis, which proceeds normally in eyes lacking ft or ex (Bennett and Harvey, 2006; Harvey et al., 2003; Tapon et al., 2002). This suggested that dco\(^2\) tissue might display differences in SWH pathway activity in a temporally restricted fashion, as with ft or ex mutant tissue.

To investigate this idea further, we analysed the cellular architecture of dco\(^1\) mosaic eyes at 44 hours APF (after developmental apoptosis has culled excess cells) by staining with antibody to Discs large (Dig; Dig1 – FlyBase). We found that interommatidial cell number in dco\(^3\) tissue was almost indistinguishable from that in wild-type tissue, similar to what has been observed in ft or ex tissue, and in contrast to sav, wts or hpo tissue (Fig. 3D) (Bennett and Harvey, 2006; Hamaratoglu et al., 2006; Jia et al., 2003; Kango-Singh et al., 2002; Pantalacci et al., 2003; Silva et al., 2006; Tapon et al., 2002; Udan et al., 2003; Willecke et al., 2006; Wu et al., 2003). To directly determine whether Dco is required for developmental apoptosis in the eye, we performed TUNEL analysis on dco\(^3\) mosaic eye discs at 28 hours APF, when apoptosis normally occurs. Apoptosis proceeded unperturbed in dco\(^3\) tissue, showing conclusively that recessive dco\(^2\) pupal retinal cells are sensitive to developmental apoptosis (Fig. 3E).

We then investigated the developmental stages at which Dco might regulate SWH pathway activity by measuring the expression of DIAP1, Ex and ban in dco\(^2\) larval and pupal eye tissue. In larval dco\(^2\) eye tissue, DIAP1 mRNA (Fig. 3F), DIAP1 protein (see Fig. S3A in the supplementary material) and ex mRNA (Fig. 3G) were elevated. As occurred in tissue lacking ft or ex, ban was only slightly elevated in dco\(^2\) tissue (Fig. 3H). In pupal eye tissues lacking dco\(^3\), DIAP1 mRNA, ex mRNA and ban were expressed at levels equivalent to wild-type tissue (Fig. 3I,J,K), as was DIAP1 protein (see Fig. S3B in the supplementary material). These data suggest that Dco controls SWH pathway activity during larval, but not pupal, eye development.

![Fig. 3. Recessive dco\(^2\) eye tissue displays elevated Yki activity during larval, but not pupal, development.](image-url)
Precocious cell proliferation in pupal eye tissue recessive for hpo, but not ft, ex or dco³

The SWH pathway controls eye size by regulating growth and proliferation rates throughout larval development by promoting cell cycle exit posterior to the morphogenetic furrow and promoting apoptosis at the mid-pupal stage of development (Harvey and Tapon, 2007). We wanted to determine whether the size difference of eyes lacking core versus non-core SWH pathway genes was due to cell cycle defects in the pupal eye, in addition to differential sensitivity to developmental apoptosis in the pupal eye. Ectopic proliferation in pupal eye tissue lacking core SWH pathway genes such as sav has been observed (Tapon et al., 2002). Inappropriate cell cycle re-entry has been observed in cells posterior to the morphogenetic furrow in third instar larval tissue lacking ft or ex, but has not been assessed later in eye development (Bennett and Harvey, 2006; Pellock et al., 2006; Silva et al., 2006; Tapon et al., 2002; Tyler and Baker, 2007; Willecke et al., 2006). To determine whether ectopic proliferation persists into pupal stages of development in tissue deficient for non-core SWH pathway genes, we assessed BrdU incorporation in tissue mosaic for hpo, ft, ex or dco³ 16 hours APF. Substantial proliferation was observed in hpo tissue, whereas ex, ft or dco³ cells were all quiescent at this stage of development (Fig. 4A-D). This shows that the gross difference in the size of eyes deficient in core versus non-core SWH pathway genes is due not only to differential sensitivity to apoptosis in the mid-pupal eye, but also to inappropriate proliferation that persists well into pupal development in tissue lacking core SWH pathway genes.

Expression of Ex, but not Ft and Dco, is temporally restricted in the developing eye

A possible explanation for the finding that recessive ft, ex or dco³ tissue does not display elevated Yki activity during pupal development is that Ft, Ex and Dco are expressed in a temporally restricted fashion in the eye. To investigate this notion, we analysed the expression of Ft, Ex and Dco in third instar larval eye discs and in pupal eye discs 28 hours APF, when apoptosis ensues. Initially, we analysed expression of Ex in tissue mosaic for ex⁴, a protein-null allele of ex, which allowed comparative expression between wild-type tissue and tissue that lacks expression of Ex altogether (Boedigheimer and Laughon, 1993). In third instar larval eye discs incubated with antibodies to Ex, a strong immunoreactive signal was detected in wild-type tissue, particularly at the apical junction (Fig. 5A). Tissue lacking ex⁴ also displayed some immunoreactivity, but this signal was far lower than that observed in wild-type tissue and is probably due to non-specificity of the antibody. In addition, no signal was detected at the apical junction of ex⁴ cells, where the majority of Ex presides (Boedigheimer and Laughon, 1993). At 28 hours APF, Ex immunoreactivity was roughly equivalent in wild-type and ex⁴ tissue, although it was marginally higher in the former (Fig. 5B). This suggested that at the pupal stage of development, Ex is expressed only at very low levels. To confirm this, we used immunoblot analysis to detect total Ex protein. We detected robust Ex expression in larval eye disc lysates, but found that expression of Ex was very low in pupal eye discs and only evident when the immunoblot was overexposed (Fig. 5C and data not shown). Actin was detected in equivalent amounts in both larval and pupal eyes, confirming even protein loading (Fig. 5C). Therefore, by using both immunofluorescence and immunoblot analysis, we can conclude that Ex is expressed at far lower levels in the developing eye at the pupal stage of development than at the larval stage.
We then analysed Ft expression in larval or pupal tissue mosaic for ft422 (Fig. 5D-E'). The nature of the ft422 allele is unclear (Rawls et al., 2002), but was suitable for our study because our antibody was incapable of detecting Ft protein in ft422 clones. In the larval eye Ft was present at apical junctions, and in pupal eyes the strongest Ft expression was evident at the borders of the secondary and tertiary pigment cells, the bristle cells and the four cone cells (Fig. 5E). Ft protein was expressed strongly at both larval and pupal stages of development, as evidenced by comparison of Ft expression in wild-type versus ft422 clones (Fig. 5D-E'). Therefore, unlike Ex, Ft expression is not temporally controlled throughout D. melanogaster eye development. This Ft antibody did not detect denatured protein in an immunoblot, precluding its use in this type of expression analysis.

To examine the expression profile of Dco throughout eye development, we used immunoblot analysis because the available antibody works more effectively in this assay than in situ (J. Price, personal communication). We found that Dco was expressed at equivalent amounts at both larval and pupal stages of eye development (Fig. 5F). Collectively, these analyses show that expression of Ex, but not Ft and Dco, is temporally controlled throughout development of the D. melanogaster eye.

Mer compensates for deficiency of Ft or Ex in the pupal eye

The ability of Ft and Ex to regulate organ size can be compensated for by another non-core SWH pathway protein, Mer, which is partially required for developmental apoptosis in the pupal retina (Pellock et al., 2006). For example, pupal retinal cell number in Mer;ex or Mer;ft double-mutant tissue is higher than in tissue lacking Mer, ft or ex alone (Hamaratoglu et al., 2006; Silva et al., 2006; Willecke et al., 2006). Therefore, to more definitively assess the regulatory roles of Ft and Ex in pupal eye development we assessed Yki activity in tissue lacking either Mer alone, or Mer together with either ex or ft. We failed to detect substantial elevation of SWH pathway activity in Mer eye clones 28 hours APF by measuring expression of DIAP1 protein, Ex protein and ban (Fig. 6A-B' and see Fig. S4 in the supplementary material). Next we assessed Yki activity at 28 hours APF in pupal eye tissue lacking both Mer and ex, or Mer and ft, by analysing expression of DIAP1 or Ex. In contrast to tissue lacking Mer or ex alone, Mer;ex double-mutant tissue displayed robust elevation of Yki activity as determined by increased DIAP1 or Ex (Fig. 6C-D'). A similar increase in Yki activity was observed in Mer;ft double-mutant tissue by analysing DIAP1 protein expression (Fig. 6E,E'). These results show that Ft and Ex can influence SWH pathway activity in the pupal eye, but that loss of these proteins can be compensated for by Mer.

In the absence of Mer, Ft represses Yki during pupal eye development independently of Dachs

Ft has been suggested to regulate Yki-dependent growth in larval imaginal disc tissues by influencing Ex localisation and/or expression (Bennett and Harvey, 2006; Silva et al., 2006; Willecke et al., 2006). Ft is also thought to control growth by affecting Dachs-mediated inhibition of Wts, as phenotypes associated with ft tissue, such as overgrowth and reduced expression of Wts, are reverted in tissue lacking both ft and dachs (Cho et al., 2006). To determine
whether Ft also influences Yki activity in the pupal eye in a Dachs-dependent fashion, we assessed DIAP1 expression in Mer;ft,dachs triple-mutant clones 28 hours APF. Interestingly, DIAP1 expression was elevated to similar levels in Mer;ft,dachs pupal eye tissue as in Mer;ft tissue, suggesting that at this stage of development Ft influences Yki activity independently of Dachs (Fig. 6F,F’).

**Mer is mislocalised in pupal eye tissue lacking hpo, but not ft or ex**

To address the mechanism by which Mer compensates for Ex and Ft in the pupal eye, we examined Mer protein expression. One possibility is that Mer protein levels are elevated in tissue lacking ft or ex, as this has been shown to occur in larval imaginal disc tissues lacking hpo or ex (Hamaratoglu et al., 2006). We confirmed this finding by analysing Mer expression in hpo mosaic larval eye discs and indeed found that Mer was elevated, which was particularly evident in the morphogenetic furrow (data not shown). Interestingly, in hpo pupal eye clones 28 hours APF, Mer protein was grossly mislocalised (Fig. 7). Normally, Mer is most prominent towards the apical region of the pupal eye at the junctions of the interommatidial cells, as well as being weakly localised in photoreceptor cells (Fig. 7) (McCartney and Feihon, 1996). In hpo clones, Mer was still most prominent in the apical domain but was no longer junctional and instead was localised diffusely throughout ommatidial clusters (Fig. 7A). This was particularly evident in larger hpo clones, whereas in smaller hpo clones and in ommatidia that closely apposed wild-type ommatidia Mer was sometimes observed no obvious changes in Mer localisation or levels in ft or ex pupal eye tissue, suggesting that the ability of Mer to compensate for ft or ex is not simply a consequence of an elevation in Mer protein levels (Fig. 7B-C’). In addition, no obvious changes in Mer localisation or levels were observed in pupal eye tissue homozygous for dco1 (Fig. 7D).

**DISCUSSION**

The SWH pathway controls D. melanogaster eye size by limiting growth during the larval stage of development and by restricting proliferation and promoting apoptosis during pupal development. Eyes lacking core SWH pathway components (e.g. sav, wts or hpo) are significantly larger than eyes lacking the non-core components ft, ex, dco or Mer (Fig. 3A-C). Owing to this disparity, it has been hypothesized that ft and ex only partially affect SWH pathway activity, whereas sav, wts and hpo have stronger effects, or, alternatively, that non-core components affect pathway activity in a temporally restricted fashion. Analysis of tissue recessive for ft, ex or dco1 revealed that Yki activity was elevated during larval eye development when tissues are actively growing and proliferating, but not during pupal development when apoptosis ensues, supporting the idea that Ft, Ex and Dco influence SWH pathway activity in a temporally restricted fashion. However, when tissue lacking both Mer and ft, or Mer and ex, was analysed, Yki activity was found to be elevated during both larval and pupal development, similar to the Yki activity profile observed in tissue lacking core SWH pathway proteins. This is consistent with previous reports showing that Mer acts in parallel to both Ft and Ex, and that these proteins can compensate for each other to control SWH pathway activity (Hamaratoglu et al., 2006; Silva et al., 2006; Willecke et al., 2006). Therefore, Ft and Ex do contribute to SWH pathway regulation in the pupal eye to ensure appropriate exit from the cell cycle and developmental apoptosis, but these functions can be executed by Mer in their absence, suggesting a degree of plasticity in the regulation of Yki activity by non-core SWH pathway proteins. The ability of Mer to compensate for Ft or Ex cannot simply be explained by compensatory increases in Mer protein in pupal eye tissues lacking ft or ex as we found Mer expression levels to be unaltered in these tissues.

**Ft can employ different modes of signal transduction throughout eye development**

Previous analyses of tissue lacking both ft and ex showed that these proteins function, at least in part, in parallel to control growth of larval imaginal discs (Feng and Irvine, 2007). Our analysis of ft,ex double-mutant tissue suggests that these proteins are likely to function together to control Yki activity in the pupal eye. Yki activity was not elevated in tissue lacking ft, ex or both genes, showing that these genes cannot compensate for each other in the pupal eye (Fig. 2K-L’). This is consistent with the notion that Ft influences the activity of downstream SWH pathway proteins by multiple mechanisms, an idea that is supported by our analysis of the requirement of the atypical myosin, Dachs, for Ft signalling in the pupal eye. During larval imaginal disc development, Ft can influence Yki activity by repressing Dachs activity, which in turn can repress the core SWH pathway protein Wts (Cho et al., 2006). Our analysis of pupal eye tissue that lacks both Mer and ft, or Mer, ft and dachs, showed that Yki activity was elevated in each scenario (Fig. 6E-F’). This shows that in the pupal eye, the ability of Ft to compensate for Mer is not reliant on Dachs, and implies that Ft can employ different modes of signal transduction throughout eye development. However, because Ft and Mer can compensate for each other we cannot formally conclude that normal signal transduction by Ft in the pupal eye occurs independently of Dachs.

**Expanded expression is controlled in a temporal fashion during eye development**

Expression of Ex is tightly controlled in response to alterations in SWH pathway activity at both the transcriptional and post-transcriptional levels (Harvey and Tapon, 2007). Interestingly, we also found that Ex expression is controlled in a temporal fashion throughout eye development; Ex is expressed at relatively high levels in the larval eye, but at very low levels in the pupal eye (Fig. 5A-C). Despite the fact that Ex expression is very low in the pupal eye, it clearly retains function at this stage of development because it can compensate for loss of Mer to restrict Yki activity (Fig. 6C-D’). The dynamic expression profile of Ex suggests that factors that influence its expression play an important role in defining overall eye size in D. melanogaster. At present, only two transcriptional regulatory proteins have been shown to influence the expression of ex: Yki and Sd. There are conflicting reports on whether Yki and Sd control basal expression of ex in larval imaginal discs (Wu et al., 2008; Zhang et al., 2008). It is clear, however, that Yki and Sd collaborate to drive ex expression when the activity of the SWH pathway is suppressed, presumably as part of a negative-feedback loop (Wu et al., 2008; Zhang et al., 2008). Despite the fact that basal ex expression is low in the pupal eye, the ex promoter is still responsive to Yki, as Ex expression is substantially elevated in pupal eye clones lacking hpo (Fig. 1D,D’ J,J’) or Mer and ex (Fig. 6D,D’). Future investigation of the ex promoter will help to clarify our understanding of the complex fashion by which expression of the ex gene is controlled, and should aid our understanding of eye size specification in D. melanogaster.
Concluding remarks
This study emphasises the complexity of the means by which the activity of core SWH pathway proteins is regulated by non-core proteins such as Ft, Ex, Mer and Dco. The signalling mechanisms employed by non-core proteins appear to differ at discrete stages of development in order to achieve appropriate organ size during the larval growth period of eye development, and to subsequently sculpt the eye by regulating apoptosis during pupal development.

Acknowledgements
We thank: Guschlbauer and H. Richardson for comments; M. Betson, S. Cohen, R. Fehon, G. Halder, I. Hariharan, B. Hay, K. Irvine, A. Laughon, F. Martin, D. Pan, J. Price, P. Sinha, N. Tapon, the Developmental Studies Hybridoma Bank, the Vienna Drosophila RNAi Centre, the Australian Drosophila Research Support Facility (www.ozdros.com) and the Bloomington Stock Center for fly stocks and antibodies; and the Peter Mac Microscopy core for assistance. K.F.H. holds Career Development Awards from the International Human Frontier Science Program Organization and the National Health and Medical Research Council of Australia. This work was supported by a Project Grant from the National Health and Medical Research Council of Australia and a Discovery Grant from the Australian Research Council.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl?vid=10.1242/dev.042309/-/DC1

References


