

# 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 (Rassf) 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 warranted 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

Cell Growth and Proliferation Laboratory, Peter MacCallum Cancer Centre, 7 St Andrews Place, East Melbourne, Victoria 3002, Australia and Department of Pathology, University of Melbourne, Parkville, Victoria 3010, Australia.

\*Author for correspondence (kieran.harvey@petermac.org)

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>42-47</sup> (Wu et al., 2003), *ft<sup>td</sup>* (Bryant et al., 1988), *ft<sup>td22</sup>* (Rawls et al., 2002), *ex<sup>MGH1</sup>* (Pellock et al., 2006), *ex<sup>e1</sup>* (Boedigheimer and Laughon, 1993), *dco*<sup>3</sup> (Zilian et al., 1999), *Mer<sup>d</sup>* (Fehon et al., 1997). Double-mutant clones were produced using *ft<sup>td</sup>,ex<sup>MGH1</sup> FRT40A* (generated by meiotic recombination), or *yki<sup>B5</sup>*, a FRT82B *yki* rescue construct (Huang et al., 2005) and either *wts<sup>X1</sup>* (Xu et al., 1995) or *dco*<sup>3</sup>. To generate double-mutant clones containing *Mer*, the *Mer* rescue construct stock *P[w<sup>+</sup> Mer<sup>+</sup>] ubi-GFP FRT40A* was used (Hamaratoglu et al., 2006; McCartney et al., 2000). Double-mutant clones for *Mer;ex* were generated using *y w Mer<sup>d</sup> FRT19A/Y; P[w<sup>+</sup> Mer<sup>+</sup>] ubi-GFP FRT40A/ex<sup>e1</sup> FRT40A; hsFLP MKRS/+* or *y w Mer<sup>d</sup> FRT19A/Y; P[w<sup>+</sup> Mer<sup>+</sup>] ubi-GFP FRT40A/ex<sup>MGH1</sup> FRT40A; hsFLP MKRS/+*. Similarly, double-mutant clones for *Mer;ft* were generated using *y w Mer<sup>d</sup> FRT19A/Y; P[w<sup>+</sup> Mer<sup>+</sup>] ubi-GFP FRT40A/ft<sup>td</sup> FRT40A; hsFLP MKRS/+*. Triple-mutant clones for *Mer;ft,dachs* were generated using *y w Mer<sup>d</sup> FRT19A/Y; P[w<sup>+</sup> Mer<sup>+</sup>] ubi-GFP FRT40A/d<sup>GC13</sup> ft<sup>td</sup> FRT40A; hsFLP MKRS/+*. Other stocks included *thi<sup>c58</sup>* (Hay et al., 1995), *ex<sup>697</sup>* (Boedigheimer and Laughon, 1993), *bantam-GFP* sensor transgene (Brennecke et al., 2003), *dco*<sup>2</sup> (Zilian et al., 1999), *yki<sup>B5</sup>* (Huang et al., 2005), *UAS-ex RNAi* (Dietzl et al., 2007), *71B-Gal4*, *y w eyFlp; FRT40A P[W+ ubi-GFP]*, *y w hsFlp; FRT40A P[W+ ubi-GFP]*, *y w hsFlp; FRT40A P[W+ arm-lacZ]*, *y w eyFlp; FRT42D P[W+ ubi-GFP]*, *y w hsFlp; FRT42D P[W+ ubi-GFP]*, *y w eyFlp; FRT82B P[W+ ubi-GFP]*, *y w hsFlp; FRT82B P[W+ ubi-GFP]*, *y w hsFlp; FRT82B P[W+ arm-lacZ]*, *y w P [W+ ubi-GFP] FRT19A* and *eyFlp, w P [miniW+ arm-lacZ] FRT19A; +; eyFlp/TM6B*.

### Immunohistochemistry

Primary antibodies used were directed against DIAP1 (from B. Hay) (Yoo et al., 2002), Ex (from A. Laughon) (Boedigheimer and Laughon, 1993), Ft (this study), Dco (from J. Price) (Muskus et al., 2007), Mer (McCartney and Fehon, 1996), Dlg and Actin (Developmental Studies Hybridoma Bank) and  $\beta$ -galactosidase (Sigma). Anti-mouse, anti-rat and anti-rabbit secondary antibodies were from Molecular Probes. Tissues were fixed in 4% paraformaldehyde and incubated with antibodies overnight at 4°C in phosphate-buffered saline containing 0.3% Triton X-100 as described (Harvey et al., 2008).

### Apoptosis assays

TUNEL assays were performed on pupal eye discs 28 hours APF, using a TMR-Red Roche Kit as previously described (Bennett and Harvey, 2006).

### Fat antibody production

GST-Fat was cloned and purified as described (Yang et al., 2002). Rabbits were inoculated with GST-Fat by IMVS Veterinary Services (Adelaide) and serum collected.

### Immunoblotting

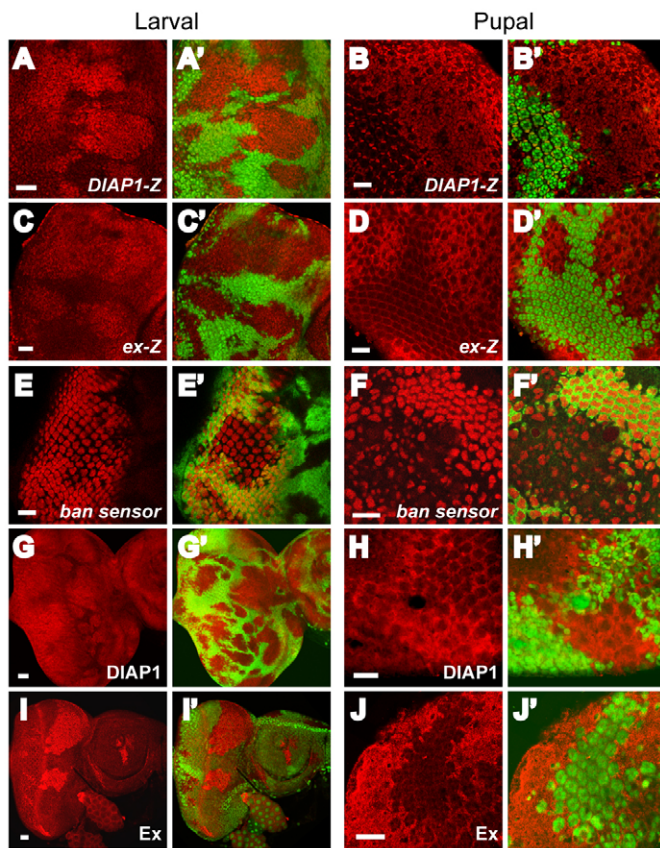
Third instar larval or mid-pupal (28 hours APF) eye imaginal discs and brains were lysed directly in protein sample buffer. Samples were subjected to SDS-PAGE, transferred to Immobilon-P membrane (Millipore) and probed with anti-Ex (Boedigheimer and Laughon, 1993), anti-Dco (Muskus et al., 2007) or anti-Actin (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 DIAP1 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 DIAP1 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 temporally 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 *DIAP1*, *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 DIAP1 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 expression of GFP or  $\beta$ -galactosidase ( $\beta$ -gal), whereas mutant tissue failed to express these proteins. In tissue lacking the core SWH pathway component *hpo*, we observed increased expression of DIAP1 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 DIAP1, 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).

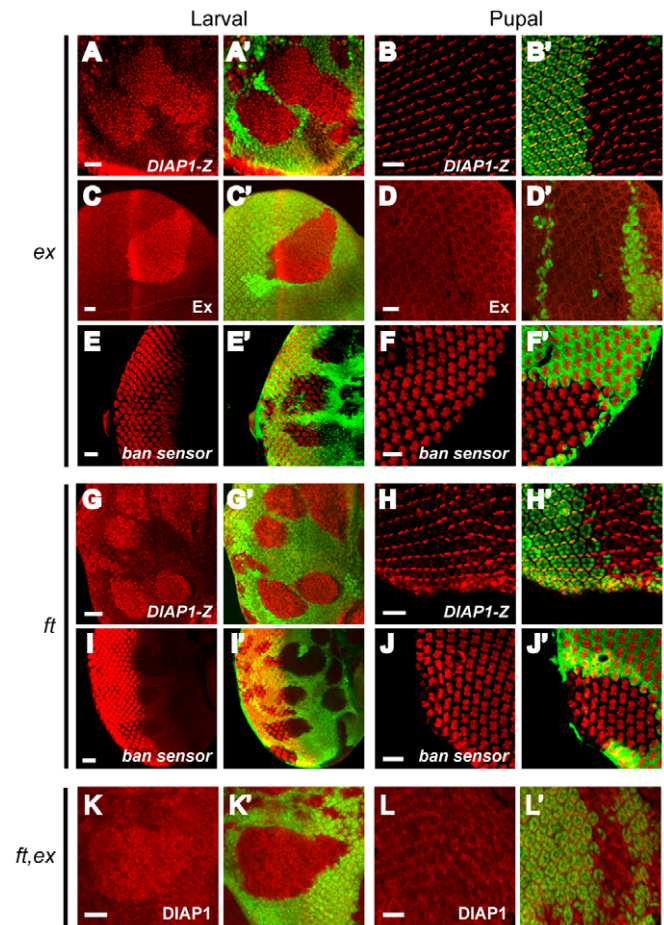


**Fig. 1. Core SWH pathway members control pathway activity throughout *Drosophila* larval and pupal eye development.**

(A-J') Third instar larval eye discs (A,C,E,G,I) or pupal eye discs 28 hours APF (B,D,F,H,J) mosaic for *hpo*<sup>42-47</sup>. Expression of *DIAP1* mRNA is reported using a *DIAP1-lacZ* enhancer trap (red in A-B'). *ex* mRNA expression is reported using an *ex-lacZ* enhancer trap (red in C-D'). *ban* expression is evident as a reduction in expression of the *ban-GFP* sensor (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. *hpo*<sup>42-47</sup> 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  $\mu$ m.

### 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



**Fig. 2. Eye tissue lacking *ft* or *ex* displays elevated Yki activity during larval, but not pupal, development.** (A-L') Third instar larval eye discs (A,C,E,G,I,K) or pupal eye discs 28 hours APF (B,D,F,H,J,L) mosaic for *ex*<sup>MGH1</sup> (A-F'), *ft*<sup>td</sup> (G-J') or *ft*<sup>td</sup>, *ex*<sup>MGH1</sup> (K-L'). Expression of *DIAP1* mRNA is reported using a *DIAP1-lacZ* enhancer trap (red in A-B', G-H'). Ex expression was detected using an antibody specific for this protein (red in C-D'). *ban* expression is evident as a reduction in expression of the *ban-GFP* sensor (red in E-F', I-J'). *DIAP1* expression was detected using an antibody specific for this protein (red in K-L'). *ex*<sup>MGH1</sup>, *ft*<sup>td</sup> and *ft*<sup>td</sup>, *ex*<sup>MGH1</sup> double-mutant clones lack expression of GFP (green in A'-D', G', H', K', L') or *lacZ* (green in E', F', I', J') and are merged with gene or protein expression images in A'-L'. Scale bars: 20  $\mu$ m.

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).

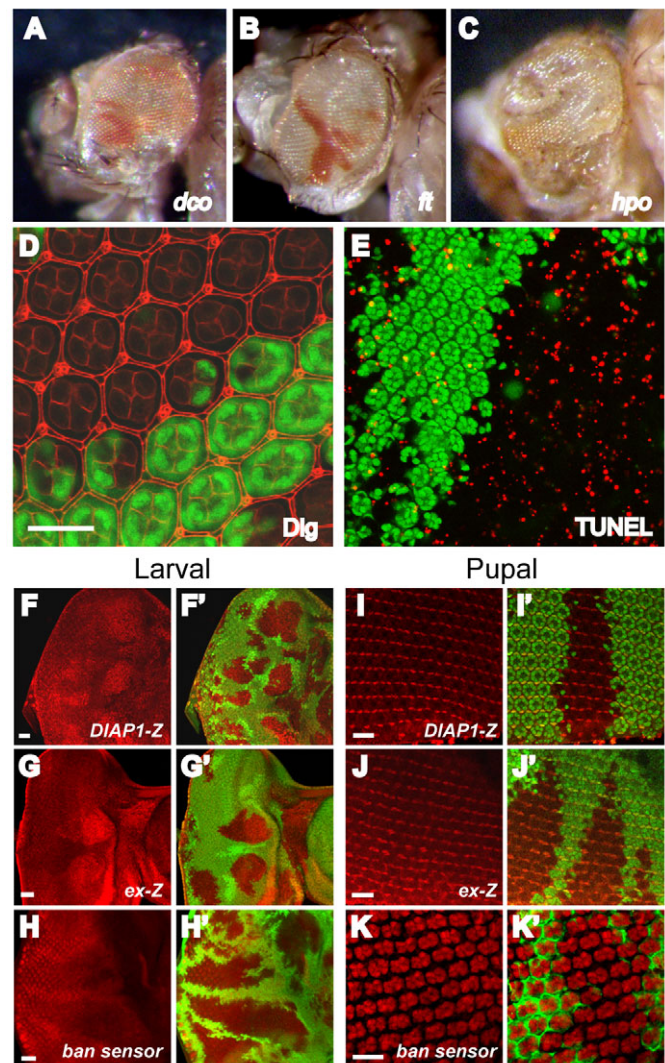
### Recessive *dco*<sup>3</sup> eye tissue displays elevated Yki activity during larval, but not pupal, development

Previously, recessive *dco*<sup>3</sup> 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*<sup>3</sup> 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*<sup>3</sup>) or transheterozygous (*dco*<sup>2</sup>/*dco*<sup>3</sup>) 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*<sup>3</sup> allele encodes a dominant-negative protein that blocks Fat phosphorylation (Badouel 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*<sup>3</sup> mutant tissue further. Initially, we examined the phenotype of *dco*<sup>3</sup> mosaic eyes generated by the eyFlp system. *dco*<sup>3</sup> 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*<sup>3</sup> tissue might display differences in SWH pathway activity in a temporally restricted fashion, as with *ft* or *ex* mutant tissue.

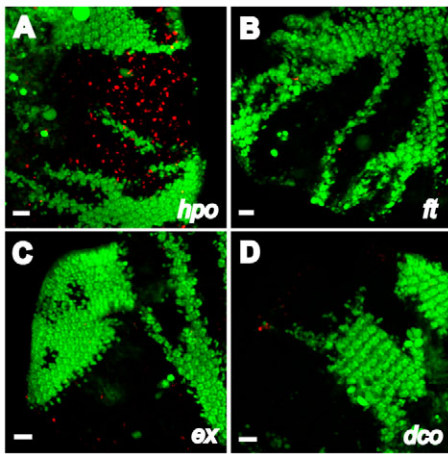
To investigate this idea further, we analysed the cellular architecture of *dco*<sup>3</sup> mosaic eyes at 44 hours APF (after developmental apoptosis has culled excess cells) by staining with antibody to Discs large (Dlg; Dlg1 – FlyBase). We found that interommatidial cell number in *dco*<sup>3</sup> 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; Harvey et al., 2003; 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*<sup>3</sup> mosaic eye discs at 28 hours APF, when apoptosis normally occurs. Apoptosis proceeded unperturbed in *dco*<sup>3</sup> tissue, showing conclusively that recessive *dco*<sup>3</sup> 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*<sup>3</sup> larval and pupal eye tissue. In larval *dco*<sup>3</sup> 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



**Fig. 3. Recessive *dco*<sup>3</sup> eye tissue displays elevated Yki activity during larval, but not pupal, development.** (A–C) Adult mosaic eyes comprising wild-type clones (red or orange) and either *dco*<sup>3</sup> (A), *ft*<sup>td</sup> (B) or *hpo*<sup>42-47</sup> (C) clones (white). Eyes mosaic for *dco*<sup>3</sup> more closely resemble the size and morphology of eyes mosaic for *ft*<sup>td</sup> than the vastly overgrown *hpo*<sup>42-47</sup> mosaic eyes. (D) Cell outlines as detected by Discs large (Dlg) protein expression (red) in a *dco*<sup>3</sup> mosaic pupal eye 44 hours APF. (E) Apoptosis in *dco*<sup>3</sup> mosaic eyes 28 hours APF as indicated by TUNEL analysis (red cells). In D and E, wild-type tissue expresses GFP (green) whereas *dco*<sup>3</sup> tissue does not. (F–K') Third instar larval eye discs (F,G,H) or pupal eye discs 28 hours APF (I,J,K) mosaic for *dco*<sup>3</sup>. *dco*<sup>3</sup> clones lack expression of GFP (F',G',I',J') or *lacZ* (H',K') and are merged with gene expression images in F'–K'. Expression of *DIAP1* mRNA is reported using a *DIAP1-lacZ* enhancer trap (red in F,F',I,I'). *ex* mRNA expression is reported using an *ex-lacZ* enhancer trap (red in G,G',J,J'). *ban* expression is reported using a *ban-GFP* sensor (red in H,H',K,K'). Scale bars: 20 μm.

elevated in *dco*<sup>3</sup> tissue (Fig. 3H). In pupal eye tissues lacking *dco*<sup>3</sup>, *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.



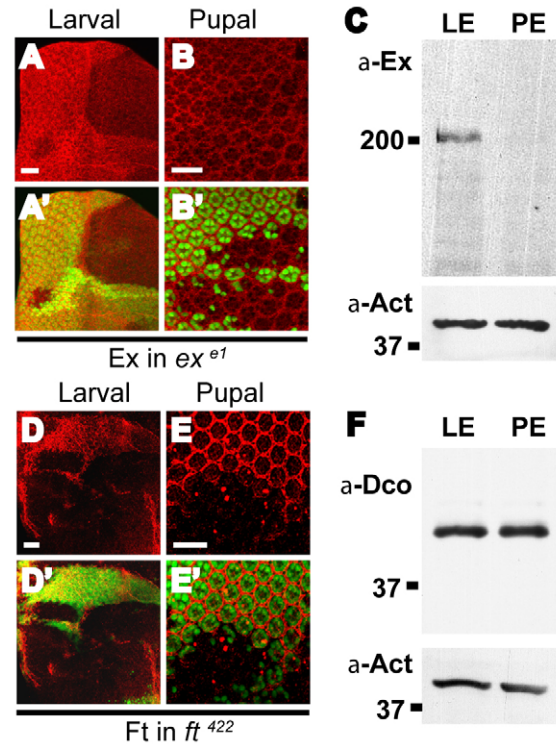
**Fig. 4. Precocious cell proliferation in pupal eye tissue recessive for *hpo*, but not *ft*, *ex* or *dco*<sup>3</sup>.** Mosaic eyes 20 hours APF comprising GFP-positive wild-type clones (green) and either (A) *hpo*<sup>42-47</sup>, (B) *ft*<sup>4d</sup>, (C) *ex*<sup>e1</sup> or (D) *dco*<sup>3</sup> clones, which fail to express GFP. Proliferating cells are BrdU positive (red). Scale bars: 20  $\mu$ m.

#### Precocious cell proliferation in pupal eye tissue recessive for *hpo*, but not *ft*, *ex* or *dco*<sup>3</sup>

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 *dco*<sup>3</sup> 16 hours APF. Substantial proliferation was observed in *hpo* tissue, whereas *ex*, *ft* or *dco*<sup>3</sup> 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.

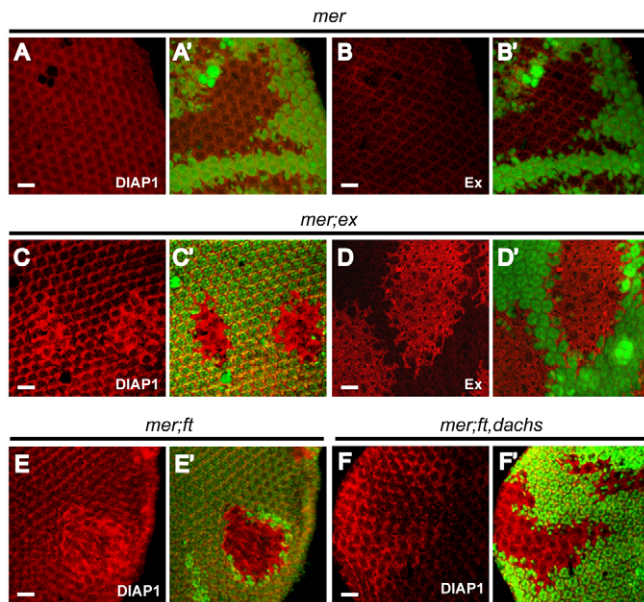
#### Expression of Ex, but not Ft and Dco, is temporally restricted in the developing eye

A possible explanation for the finding that recessive *ft*, *ex* or *dco*<sup>3</sup> 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 *ex*<sup>e1</sup>, a protein-null allele of *ex*, which allowed comparative expression between wild-type tissue and tissue that lacks expression of Ex altogether



**Fig. 5. Ex, but not Ft and Dco, is expressed in a temporally restricted fashion in the developing *D. melanogaster* eye.** (A-B') Ex protein expression (red) in *ex*<sup>e1</sup> mosaic larval (A, A') or pupal (B, B') eye discs. Wild-type tissue expresses GFP (green in merged images, A', B'), whereas mutant tissue does not. (C) Immunoblot analysis of Ex protein in wild-type imaginal discs from third instar larval eye discs (LE) or pupal eye discs 28 hours APF (PE). Actin (Act) expression was detected to ensure even loading of protein lysates; molecular mass markers (kDa) are indicated. (D-E') Ft protein expression (red) in *ft*<sup>422</sup> mosaic larval (D, D') or pupal (E, E') eye discs. Wild-type tissue expresses GFP (green in merged images, D', E'), whereas mutant tissue does not. (F) Immunoblot analysis of Dco protein in wild-type imaginal discs from third instar larval eye discs or pupal eye discs 28 hours APF. Scale bars: 20  $\mu$ m.

(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 *ex*<sup>e1</sup> 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 *ex*<sup>e1</sup> cells, where the majority of Ex resides (Boedigheimer and Laughon, 1993). At 28 hours APF, Ex immunoreactivity was roughly equivalent in wild-type and *ex*<sup>e1</sup> 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.



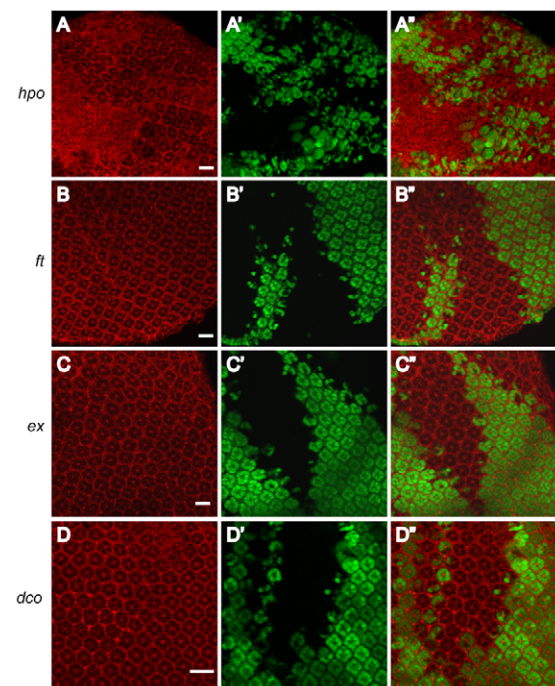
**Fig. 6. Ft, Ex and Mer all influence SWH pathway activity in the pupal eye.** Pupal eye discs 28 hours APF mosaic for (A-B') *Mer*, (C-D') *Mer<sup>A</sup>;ex<sup>e1</sup>*, (E,E') *Mer<sup>A</sup>;ft<sup>d</sup>* or (F,F') *Mer<sup>A</sup>;ft<sup>d</sup>;dachs<sup>13</sup>*. Expression of DIAP1 was detected using an antibody directed against this protein (red in A,A',C',E-F'). Ex protein was detected using an Ex-specific antibody (red in B,B',D,D'). In each case, mutant clones lack expression of GFP (green) and are merged with protein expression in A-F'. Scale bars: 20 μm.

We then analysed Ft expression in larval or pupal tissue mosaic for *ft<sup>422</sup>* (Fig. 5D-E'). The nature of the *ft<sup>422</sup>* allele is unclear (Rawls et al., 2002), but was suitable for our study because our antibody was incapable of detecting Ft protein in *ft<sup>422</sup>* 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 *ft<sup>422</sup>* 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



**Fig. 7. Mer is mislocalised in pupal eye tissue lacking *hpo*.** Pupal eye discs 28 hours APF mosaic for (A-A') *hpo<sup>42-47</sup>*, (B-B') *ft<sup>d</sup>*, (C-C') *ex<sup>e1</sup>* or (D-D') *dco<sup>3</sup>*. Expression of Mer was detected using an antibody directed against this protein (red in A,B,C,D). Mutant clones lack expression of GFP (green in A'-D') and are merged with protein expression images in A''-D''. Scale bars: 20 μm.

*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 Fehon, 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 distributed in the normal pattern, i.e. junctional (Fig. 7A). The functional significance of Mer mislocalisation in *hpo* pupal eye clones is unclear but suggests the interesting possibility that Hpo regulates Mer localisation in the pupal eye. By contrast, we 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 *dco*<sup>3</sup> (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 *dco*<sup>3</sup> 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.

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## Competing interests statement

The authors declare no competing financial interests.

## Supplementary material

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