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Article

# The Fat Cadherin Acts through the Hippo Tumor-Suppressor Pathway to Regulate Tissue Size

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## Summary

**Background:** The Hippo tumor-suppressor pathway has emerged as a key signaling pathway that controls tissue size in *Drosophila*. Merlin, the *Drosophila* homolog of the human Neurofibromatosis type-2 (NF2) tumor-suppressor gene, and the related protein Expanded are the most upstream components of the Hippo pathway identified so far. However, components acting upstream of Expanded and Merlin, such as transmembrane receptors, have not yet been identified.

**Results:** Here, we report that the protocadherin Fat acts as an upstream component in the Hippo pathway. Fat is a known tumor-suppressor gene in *Drosophila*, and *fat* mutants have severely overgrown imaginal discs. We found that the overgrowth phenotypes of *fat* mutants are similar to those of mutants in Hippo pathway components: *fat* mutant cells continued to proliferate after wild-type cells stopped proliferating, and *fat* mutant cells deregulated Hippo target genes such as *cyclin E* and *diap1*. Fat acts genetically and biochemically upstream of other Hippo pathway components such as Expanded, the Hippo and Warts kinases, and the transcriptional coactivator Yorkie. Fat is required for the stability of Expanded and its localization to the plasma membrane. In contrast, Fat is not required for Merlin localization, and Fat and Merlin act in parallel in growth regulation.

**Conclusions:** Taken together, our data identify a cell-surface molecule that may act as a receptor of the Hippo signaling pathway.

## Introduction

During development, the number of cells in growing tissues is tightly regulated to ensure generation of organs of proper size [1–3]. Cell number is controlled by regulating the generation of new cells through cell proliferation and by regulating apoptosis to eliminate excess or damaged cells [1–3]. The Hippo (Hpo) tumor-suppressor pathway has emerged as a key signaling pathway that controls tissue size in *Drosophila* (reviewed in [4]). Hpo signaling restricts tissue size by promoting apoptosis and cell-cycle arrest, and animals carrying clones of cells mutant for *hpo* develop severely overgrown adult structures. Several components of the Hpo pathway have been discovered, and a signal transduction pathway from the plasma membrane to the nucleus has begun to emerge [5–16]. Merlin (Mer) and Expanded (Ex) are currently the most upstream components known in the Hpo pathway [5]. Mer and Ex are related 4.1, Ezrin, Radixin, Moesin (FERM)-domain-containing adaptor proteins localized to the plasma membrane [17, 18], where they are thought to transduce a growth-regulatory signal to Hpo [5], a serine/threonine kinase of the Sterile-20 family [8–12]. Hpo, together with its cofactor Salvador (Sav) [6, 7], then causes phosphorylation and thereby activation of Warts (Wts), a NDR-type kinase [13, 14]. Wts, together with its cofactor, Mats (Mob as a tumor suppressor) [15], phosphorylates and regulates the activity of Yorkie (Yki), a transcriptional coactivator [16]. Ex, Mer, Hpo, Sav, Wts, and Mats are negative regulators of growth, and mutations in these genes result in dramatically overgrown tissues containing an excess number of cells. Yki, on the other hand, is a positive regulator of growth, and overexpression of Yki causes severe overgrowths that resemble the loss-of-function phenotypes of the other pathway members, whereas cells mutant for *yki* grow poorly [16]. Wts negatively regulates the transcriptional activity of Yki in a cell-culture-based assay, possibly through phosphorylation [16]. It was thus postulated that Wts promotes cell-proliferation arrest and apoptosis through the inactivation of Yki, which otherwise induces the expression of genes that drive cell proliferation and cell survival [16].

Here, we identify the Fat (Ft) protocadherin as an upstream component in the Hpo signaling pathway. Ft acts as a tumor suppressor to restrict imaginal-disc growth, and imaginal discs from *ft* mutants are much larger than wild-type discs [19, 20]. This is because mutant cells proliferate faster than normal and fail to arrest proliferation when discs have reached their proper size [19, 21, 22]. In addition to its tumor-suppressor function, *ft* is also required for the establishment of normal planar-cell polarity (PCP) in the eye, wing, and abdomen [23–28] as well as for proximal-distal patterning of appendages [19, 29].

For all three functions, Ft interacts with Dachsous (Ds), a related protocadherin [30]. Like Ft, Ds is required for PCP, proximal-distal patterning, and growth control [26–35]. Ft and Ds are atypical cadherins with large extracellular and cytoplasmic domains that are different from those of classical cadherins [20, 30]. Ft and Ds preferentially bind each other, and it has been proposed that

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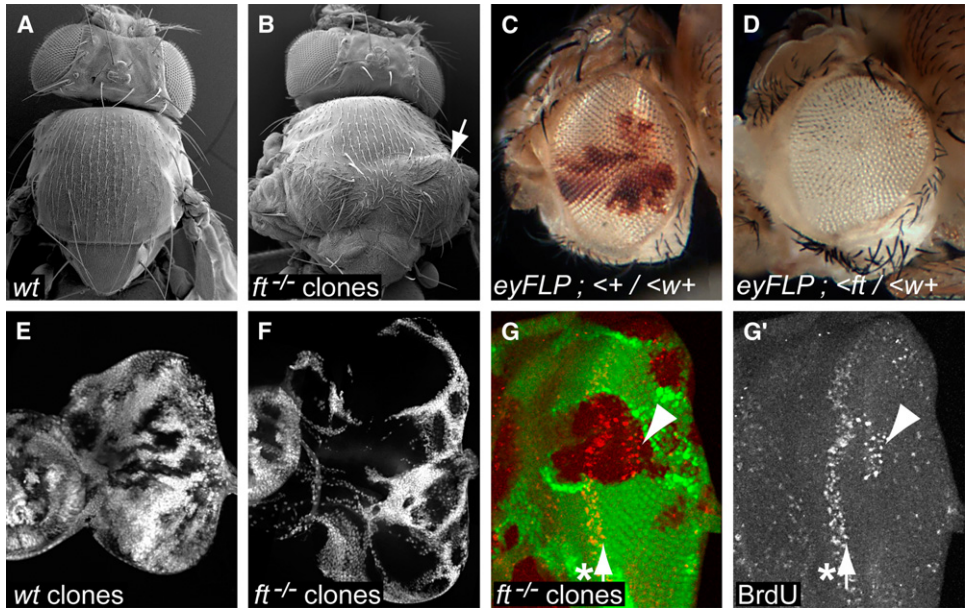


Figure 1. Fat Regulates Tissue Size and Cell-Cycle Arrest

(A and B) Scanning electron micrographs of a wild-type fly thorax and a thorax with $ft^{422}$ mutant clones, which resulted in massive tissue overgrowths (arrow).

(C) Fly with eyes mosaic for a mutation in the *white* ($w^-$) gene. For this experiment, we used the eye-specific FLP driver *ey-FLP* to induce mitotic recombination in most of the cells in the developing eye. For increasing the area of the $w^-$ cell clones, a cell-lethal mutation on the homologous $w^+$ chromosome was used to eliminate twin clones. The resulting eye was composed of about 70% $w^-$ cells.

(D) Fly with a mosaic eye induced by the same method as in (C). This fly, however, carried a $ft^{G\text{-}rv}$ mutation on the $w^-$ chromosome. The $ft^{G\text{-}rv}$ mutant $w^-$ cells made up nearly the entire eye, and these cells had thus outcompeted the red (wild-type) cells.

(E and F) Eye imaginal discs from third-instar larvae containing *wt* (E) and $ft^{fd}$ (F) mutant clones that were marked by the absence of GFP expression (gray). Clones were induced by using *ey-FLP*.

(G) Eye imaginal disc containing $ft^{fd}$ mutant clones. This disc was labeled for BrdU incorporation (red in [G] and gray in [G′]). Wild-type cells arrest in G1 in the morphogenetic furrow (asterisks), and nondifferentiating cells go through one synchronous S phase in the second mitotic wave (arrows). $ft^{fd}$ mutant cell clones showed ectopic cell proliferation posterior to the second mitotic wave (arrowheads). Anterior is to the left in all discs.

they act as receptor (Ft) and ligand (Ds) [27, 28, 32, 34, 35]. However, Ds also has Ft-independent functions in growth control because imaginal discs from *ft,ds* double mutants show more severe overgrowths than those from homozygous *ft* or *ds* mutants [32]. Ft and Ds thus act in parallel to regulate tissue size.

Notably, overexpression of a version of Ft that lacks its extracellular domain is sufficient to rescue the growth and PCP defects exhibited in *ft* mutant discs [32]. These findings support a model in which Ft acts as a receptor with its intracellular domain mediating the PCP and growth-control signals [32]. Ft may mediate its effects on PCP via the transcriptional corepressor Grunge (Atrophin), which binds to the intracellular domain of Ft [36]. The unconventional myosin Dachs (D) is required for the growth and proximal-distal patterning functions of Ft; however, the role of D in Ft signaling is not known [29, 37]. The pathway through which Ft acts as a tumor-suppressor gene to regulate tissue size is thus only poorly understood.

Here, we provide evidence that Ft acts through the Hpo pathway to regulate tissue size. We found that the overgrowth phenotypes of *ft* mutations are similar to those of mutations in Hpo pathway components. Ft is required for the localization of Ex to the plasma membrane and acts genetically and biochemically upstream of Ex, Hpo, Wts, and Yki. Taken together, our data place Ft upstream of Ex and identify a cell-surface molecule that may act as a receptor of the Hpo signaling pathway.

## Results

### Mutations in *fat* Deregulate Cell Proliferation

It has long been known that *ft* mutant discs are severely overgrown because mutant cells overproliferate [19]. Indeed, clones of cells mutant for *ft* produced dramatic outgrowths in diverse adult structures such as antennae, thoraxes, wings, and legs (Figures 1A and 1B and not shown). In addition to growing beyond normal tissue size, *ft* mutant cells have a growth advantage over wild-type cells. *ft* mutant cells, marked by the absence of pigmentation (mutant for *white* [$w^-$]), outcompeted red ($w^+$) pigmented wild-type cells and overtook nearly the entire eye, although only about half of the cells were initially made mutant for *ft* through *ey-FLP*-induced mitotic recombination (Figure 1D). In contrast, clones of cells that just lacked pigmentation had no growth advantage and allowed red cells to contribute to the adult eye (Figure 1C). Thus, *ft* mutant cells outcompeted their wild-type siblings, resulting in eyes that were nearly entirely composed of *ft* mutant cells. This growth advantage of *ft* mutant cells is already observed at the third-instar stage, when *ey-FLP*-induced *ft* mutant clones occupied nearly the entire disc tissues in contrast to

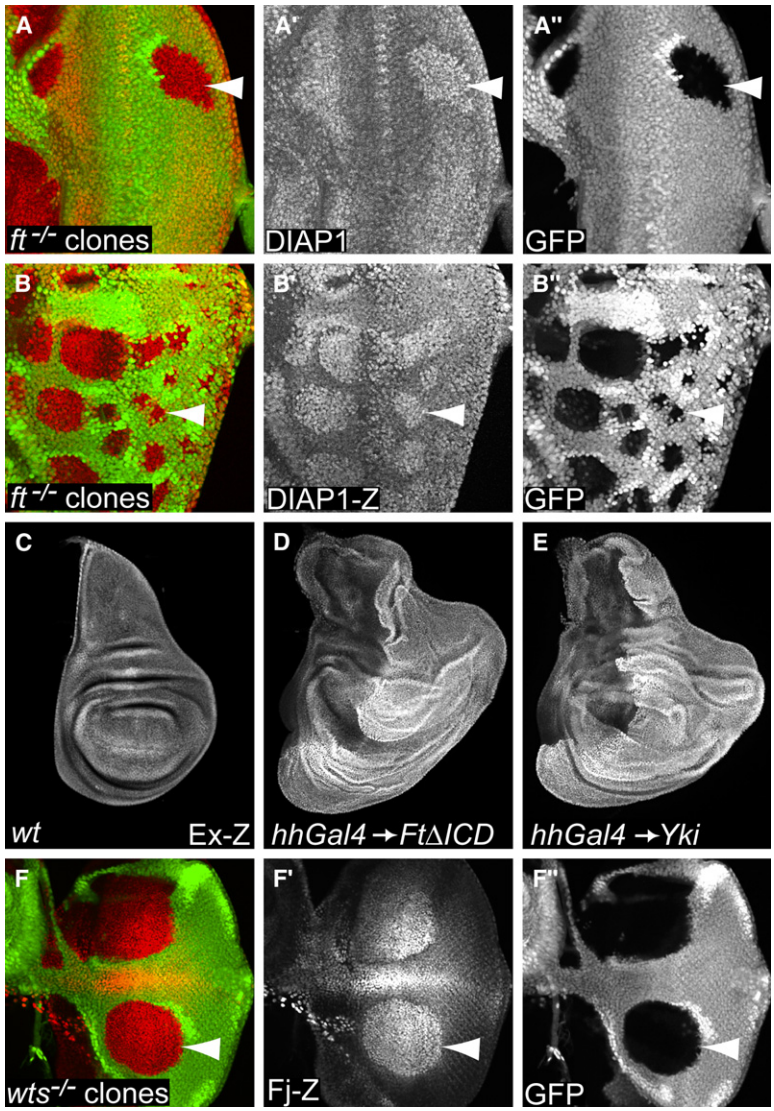


Figure 2. Fat Regulates Hippo Target Genes

(A and B) Third-instar eye imaginal discs containing $ft^{fd}$ mutant clones marked by the absence of GFP expression (green in [A] and [B] and grayscale in [A″] and [B″]). (A–A″) $ft^{fd}$ mutant clones upregulated DIAP1 levels (red in [A], grayscale in [A′]). Arrowhead points to a mutant area. (B–B″) $ft^{fd}$ mutant clones (arrowhead) upregulated the expression of a *lacZ* enhancer-trap insertion in the *diap1* gene (red in [B], grayscale in [B′]).

(C–E) Discs stained to detect βGal expression from a *lacZ* enhancer-trap insertion into the *ex* gene (grayscale) in a wild-type background (C), in wings disc that expressed FtΔICD (D), and Yki (E) in the posterior compartment. Both, FtΔICD and Yki expression caused overgrowth of the posterior compartment and induction of *ex-lacZ* expression.

(F) $wts^{x1}$ clones, marked by the absence of GFP expression (green in [F], grayscale in [F″]), induced the expression of a *fj-lacZ* reporter (red in [F], grayscale in [F′]) in an eye imaginal disc. Anterior is to the left and dorsal is up for all discs.

wild-type clones, which occupied less than half of the discs (Figures 1E and 1F). Similarly, *ft* mutant cells have a growth advantage in the developing wing [22]. Ft is thus a general growth regulator that is required to restrict the rate of cell proliferation and the size of adult structures.

Defects in the regulation of cell proliferation are readily detectable in the developing eye, where posterior to the second mitotic wave all cells cease to proliferate [38, 39]. However, only a fraction of these cells have been determined and begin to differentiate as photoreceptor cells, whereas the remaining cells are still undetermined and will later form the cone, pigment, and bristle cells [38, 39]. In contrast to wild-type cells, *ft* mutant cells failed to arrest the cell cycle after the second mitotic wave and instead continued to proliferate, as evidenced by ectopic Bromodeoxyuridine (BrdU) incorporation, which marks cells in S phase. (Figure 1G, arrowhead). Double labeling with the neuronal marker ELAV showed that ectopic proliferation occurred only in developmentally uncommitted cells, but not in differentiating photoreceptor cells (Figure S1 in the Supplemental Data available online). We conclude that Ft is required for cell-proliferation arrest of uncommitted precursor cells.

## Fat Regulates Hippo Target Genes

The adult overgrowth phenotypes, the growth advantage of mutant cells, and the continued proliferation of uncommitted precursor cells are characteristic for mutations in Hpo pathway components [5]. These similarities thus prompted us to test whether Ft regulates genes known to be regulated by Hpo signaling. We found that *ft* mutant clones cell-autonomously upregulated the expression of DIAP1 (Figure 2A), similar to cells lacking Hpo activity [5–12]. This regulation was at the level of transcription, because *ft* mutant cells upregulated the expression of a *lacZ* reporter for the *diap1* gene (Figure 2B). *ft* mutant cells also upregulated the expression of Cyclin E, which is typical for *hpo* mutant cells (Figure 3E) [8–12]. The regulation of Cyclin E and DIAP1 expression are likely important downstream effects of Ft for the regulation of cell number and tissue size.

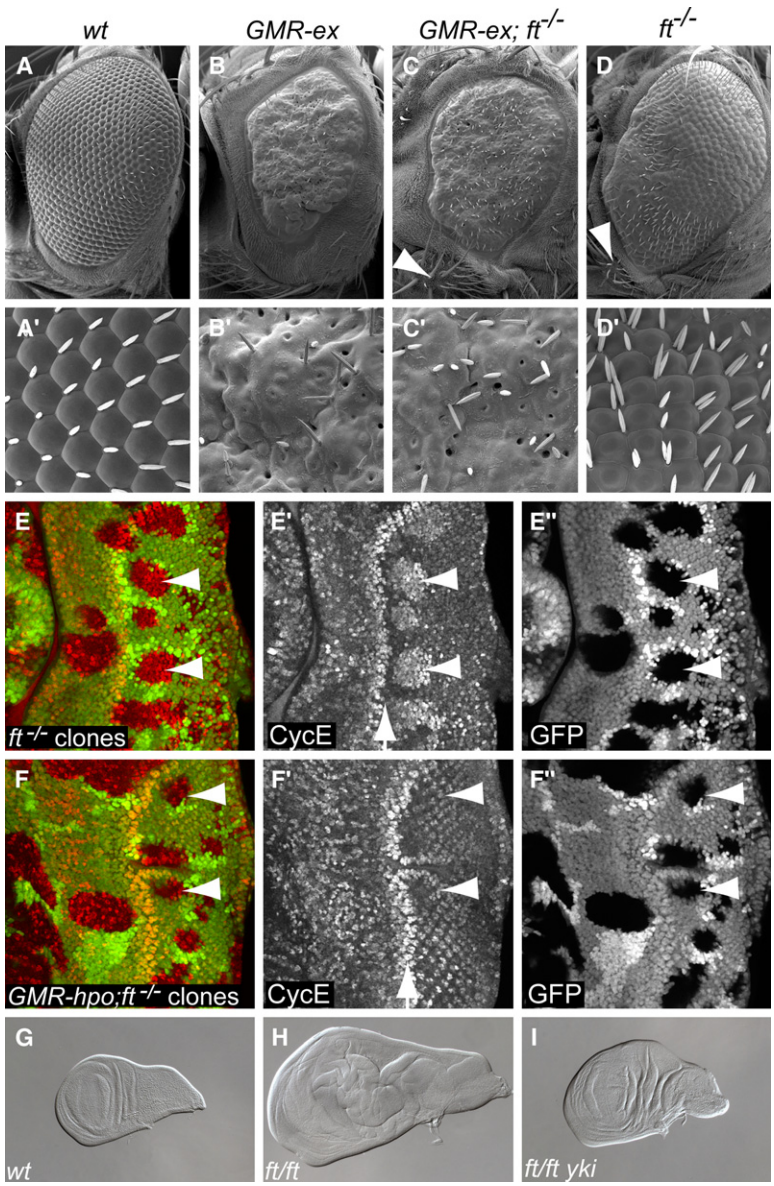


Figure 3. Fat Acts Genetically Upstream of Known Hippo Pathway Components

Panels (A)–(D) show SEM images of eyes of adult flies. The genotypes of the animals are indicated above the panels. *GMR-ex* refers to *GMR-Gal4*-driven overexpression of a *UAS-ex* transgene. (A′)–(D′) show higher-magnification images of the panels above them. *GMR-Gal4* drives expression of the *UAS-ex* transgene in the developing eye behind the morphogenetic furrow. *ft* mutant heads were generated via *ey-FLP*-mediated mitotic recombination to generate heads that were basically entirely mutant for $ft^{422}$. Overexpression of Ex caused reduced and rough eyes (B and B′), whereas loss of *ft* caused eye overgrowth and duplications of ventral vibrissae (arrowhead in [D]). Ex expression still caused small and rough eyes in *ft* mutant heads (C and C′). Because Ex was only expressed in the eye, it did not suppress the head overgrowth and extra ventral vibrissae phenotypes of *ft* mutant heads (arrowhead in [C]).

(E) $ft^{422}$ mutant clones cell-autonomously upregulated the expression of Cyclin E (arrowheads), most conspicuously behind the second mitotic wave (arrows). Mutant clones were marked by the absence of GFP expression (green in [E] and grayscale in [E″]) and Cyclin E expression is shown in red in (E) and in grayscale in (E′).

(F) $ft^{422}$ mutant clones in an eye disc that overexpressed Hpo, driven by *GMR-Gal4*, posterior to the morphogenetic furrow. Mutant clones posterior to the second mitotic wave did not induce Cyclin E expression, unlike $ft^{422}$ mutant clones in a wild-type background (E). Arrowheads point to mutant areas and anterior is to the left. Coloring is the same as in (E).

(G–I) Third-instar wing imaginal discs of the following genotypes: (G) *wt*, (H) $ft^{fd}/ft^{G\text{-}rv}$, and (I) $ft^{fd}$ $yki^{B5}/ft^{G\text{-}rv}$. Removal of one copy of the *yki* gene significantly suppressed the overgrowth phenotypes of *ft* mutant imaginal discs.

We have recently reported that Hpo signaling suppresses the expression of the upstream components Ex and Mer in a negative feedback loop [5]. This regulation of *ex* and *mer* expression is independent of cell type and is observed in multiple imaginal discs, indicating an intimate connection between Hpo signaling and transcriptional regulation of *ex* and *mer*. We thus wanted to assay the expression of a *lacZ* enhancer-trap insertion in the *ex* gene in *ft* mutant cells. However, because *ex* and *ft* are located on the same chromosome arm and because *ex-lacZ* is mutant for *ex*, we could not simply assay *ex-lacZ* expression in *ft,ex-lacZ* double-mutant clones because such clones would already upregulate *lacZ* expression as a result of loss of Ex function. We thus assayed *ex-lacZ* expression in discs that were homozygous for *ft* and heterozygous for *ex-lacZ.* To visualize differences between *ft* mutant and wild-type cells in the same disc, we rescued Ft function in the posterior compartment by *hh-Gal4*-driven Ft expression. We found that anterior *ft* mutant cells had elevated levels of *lacZ* expression compared to posterior, rescued cells (Figure S2). In addition, overexpression of a dominant-negative version of Ft that lacks the intracellular domain (FtΔICD, [32]) in the posterior compartment induced overgrowth and *ex-lacZ* expression (Figures 2C and 2D) in the posterior compartment similar to the effects of Yki overexpression (Figure 2E), which is known to induce the expression of Hpo target genes [16]. In summary, our experiments show that Ft regulates the expression of *cyclin E*, *diap1*, and *ex*, known downstream target genes of the Hpo pathway.

Next we asked whether Hpo regulates the expression of known Ft downstream genes. Ft regulates the expression of *four jointed* (*fj*), which encodes a Golgi-associated protein that may regulate the activity of Ft and Ds [23, 24, 28]. *fj* is expressed in gradients in eye and wing discs, and *ft* mutant clones derepress *fj* expression [28]. Similarly, we found that *wts* mutant clones upregulated the expression of a *fj-lacZ* reporter (Figure 2F). Hpo signaling thus regulates the expression of a known Ft target gene.

### Fat Acts Genetically Upstream of Known Hippo Pathway Components

The striking similarities of the *ft* and *hpo* mutant phenotypes and the shared target genes suggested that Ft acts through Hpo signaling to regulate tissue size. To test this hypothesis genetically, we performed epistasis tests between mutations in *ft* and gain-of-function situations for other components of the Hpo pathway. If Ft acts upstream in the Hpo pathway, then hyperactivation of downstream components may rescue growth defects of *ft* mutant cells.

In a first set of experiments, we asked whether hyperactivation of Hpo signaling through overexpression of Ex was epistatic to loss of *ft* function. Overexpression of Ex hyperactivates Hpo signaling, causing a reduction in cell proliferation and induction of apoptosis, phenotypes opposite to those of Hpo loss of function [5, 8–10, 12, 40, 41]. Overexpression of Ex during eye development thus severely disrupted eye development and resulted in small and rough eyes (Figures 3A and 3B) [5, 40, 41]. On the other hand, removal of Ft function in the entire head resulted in overgrown heads with slightly bigger but deformed eyes and duplications of ventral vibrissae (Figure 3D). Overexpression of Ex in the developing eyes of *ft* mutant heads caused small and rough eyes, very similar to the overexpression of Ex in a wild-type background (Figure 3C). Loss of Ft function thus did not rescue the Ex-induced phenotypes. Rather, the overgrowth observed in *ft* mutant eyes was suppressed by overexpressed Ex. *ft* mutant phenotypes, such as extra vibrissae, were still observed in the head cuticle, where Ex was not overexpressed (Figure 3C, arrowhead). The actions of Ex are thus epistatic to the loss of Ft function.

Next, we asked whether hyperactivation of Hpo was sufficient to rescue the transcriptional upregulation of Cyclin E expression in *ft* mutant cell clones. To do this experiment, we used the *GMR-Gal4* driver to overexpress Hpo specifically posterior to the morphogenetic furrow. *ft* mutant clones robustly induced ectopic Cyclin E expression posterior to the second mitotic wave (Figure 3E, arrowheads). Hyperactivation of Hpo posterior to the furrow suppressed the upregulation of Cyclin E in *ft* mutant clones (Figure 3F arrowheads). Hpo activation is thus sufficient to rescue transcriptional defects in *ft* mutant cells.

Finally, removal of one copy of the *yki* gene significantly suppressed the overgrowth phenotypes of *ft* mutant imaginal discs (Figures 3G–3I). Notably, nearly all $ft^{fd}\ yki^{B5}/ft^{G\text{-}rv}$ mutant animals developed into adults, although the adults could not hatch from the pupal case because they still had some leg defects and head overgrowths. In contrast, all $ft^{fd}/ft^{G\text{-}rv}$ mutant animals died during the early stages of pupal development and showed severely overgrown imaginal disc derivatives. Together, our results suggest that Ex, Hpo, and Yki act downstream of Ft and support a model in which Ft acts upstream as a receptor in the Hpo pathway.

### Fat Regulates Warts Phosphorylation and Yorkie Activity

In order to more directly test the hypothesis that Ft acts as an upstream component in the Hpo pathway, we investigated whether Ft affected Wts phosphorylation and the transcriptional activity of Yki. Previous analyses showed that Hpo induces phosphorylation of Wts, and that the phosphorylation status of Wts is a readout for Hpo pathway activity [5, 10]. In S2 cells, overexpression of Hpo, Sav, Ex, and Mer induces phosphorylation of Wts, which is visualized as a shift in Wts mobility [5, 10]. We found that overexpression of full-length Ft did not affect the mobility of Wts. However, expression of Ft molecules that lacked the extracellular domain (FtΔECD) induced a Wts shift similar to the effects of Ex and Mer or Hpo and Sav (Figure 4A) [5, 10]. This shift was indeed the result of phosphorylation, because it was reversed by phosphatase treatment (Figure 4A). In addition, depletion of Hpo or Ex by RNAi abrogated the induction of Wts phosphorylation by FtΔECD (Figure 4B). Endogenously expressed Hpo and Ex are thus required for FtΔECD to induce phosphorylation of Wts.

The phosphorylation of Wts results in the activation of the Wts kinase. Activated Wts then phosphorylates Yki and suppresses its transcriptional-activator function [16]. Huang et al. have established an S2-cell-based assay that reproduces the regulation of Yki by the Hpo signaling pathway [16]. In this assay, Yki is fused to the Gal4-DNA binding domain which recruits Yki to the promoter of a *UAS-luciferase* reporter construct. The transcriptional-activator function of Yki then induces expression of the *luciferase* reporter. This activity of Yki is suppressed by activation of the Hpo pathway, for example by coexpression of Hpo, Sav, and Wts [16] or by coexpression of the upstream components Ex and Mer [5]. We found that coexpression of full-length Ft had little effect on Yki activity in this assay (Figure 4C). However, expression of the truncated version of Ft, FtΔECD, suppressed Yki activity similarly to overexpression of Ex and Mer (Figure 4C, [5]). This effect is specific, because the expression of FtΔECD, as with Ex and Mer, did not affect the activity of full-length Gal4 (Figure 4D).

We next asked whether the suppression of Yki activity by FtΔECD required the Hpo and Wts kinases. Indeed, knockdown of Wts or Hpo expression by RNAi completely rescued the suppressing effects of FtΔECD and restored the levels of Yki activity to those in control cells that were treated with *lacZ* RNAi (Figure 4E). Importantly, knockdown of Wts or Hpo did not increase the activity of Yki-Gal4 in cells that did not express the FtΔECD (Figure 4E). This result argues against Ft acting in a parallel pathway and supports a model in which Ft signals through Hpo and Wts to regulate the activity of Yki. In summary, our experiments show that Ft regulates the activity of Yki, currently the most downstream component known in the Hpo signal transduction pathway.

Overexpression of the truncated FtΔECD but not the full-length form of Ft acted as a dominantly activated Ft also in vivo. Overexpression of full-length Ft in developing wings caused wing foreshortening, similar to hypomorphic *ft* mutations ([32] and data not shown). Expression of FtΔECD, however, caused small and narrow wings that resembled the phenotypes caused by Hpo overexpression ([8, 10], Figures 4F and 4G). This phenotype was suppressed by removing one copy of *wts* (Figure 4H). Overexpression of FtΔECD thus appears to mimic active Ft and to activate the Hpo pathway.

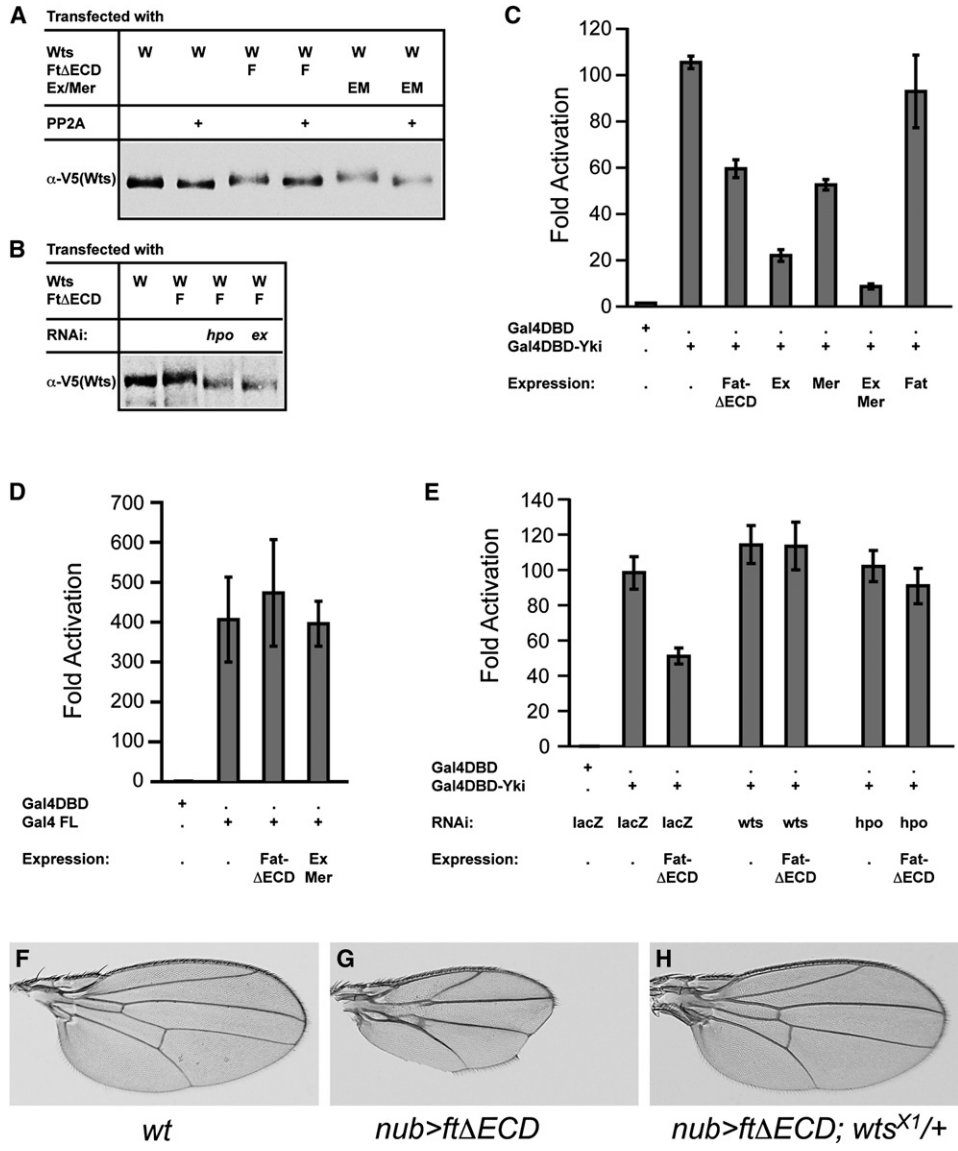


Figure 4. Fat Regulates Warts Phosphorylation and Yorkie Activity

(A and B) Ft overexpression induced Wts phosphorylation. Western blots to detect V5-tagged Wts protein in lysates from S2 cells overexpressing different combinations of proteins as indicated on top of the panels. Expression of FtΔECD induced a shift in Wts migration similar to the effects of other Hpo pathway components. This shift was reverted by PP2A treatment (A) or protein knockdown of Ex and Hpo (B).

(C) The coactivator activity of Yki was negatively regulated by FtΔECD but not by full-length Ft. S2 cells were transfected with *UAS-luc* (Gal4-responsive *luciferase* reporter) plasmid along with the indicated plasmids. The plots show the levels of induced Luciferase activities that were normalized to the level induced by the Gal4 DNA binding domain (Gal4DBD) alone (left-most bar). Error bars in (C), (D), and (E) represent standard deviations from three independent transfections.

(D) The transcriptional-activator function of the full-length Gal4 was not affected by expression of FtΔECD or Mer and Ex.

(E) The suppression of Yki-Gal4 activity by FtΔECD required Hpo and Wts. S2 cells were transfected with the indicated plasmids and treated with RNAi targeting *wts*, *hpo*, or *lacZ* as a control for comparison. Knockdown of Wts and Hpo completely suppressed the actions of FtΔECD on Yki-Gal4 activity.

(F–H) Images of wings from adults of the indicated genotypes. *nub>ft⊿ECD* refers to *nub-Gal4*-driven overexpression of a *UAS- ft⊿ECD* transgene. Overexpression of *ft⊿ECD* caused a small-wing phenotype (G). Flies that were additionally heterozygous mutant for $wts^{x1}$ showed a suppression of the growth defects (H).

## Fat Is Required for Expanded Membrane Localization

Ft is a transmembrane protein, and it may thus act as a receptor of the Hpo pathway. Ex and Mer, which act upstream of Hpo, localize to the apical-lateral plasma membrane similar to Ft ([17, 18, 28], not shown). This co-localization raised the possibility that Ft, Ex, and Mer may affect each other's localization. However, *ex* and *mer* were not required for correct localization of Ft, and *ex;mer* double-mutant cells had increased amounts of Ft at the membrane ([42] and data not shown). In contrast, Ex was largely absent from the plasma membrane in *ft* mutant cells in a cell-autonomous manner (Figure 5A). We found that wild-type and *ft* mutant wing discs expressed comparable levels of Ex protein

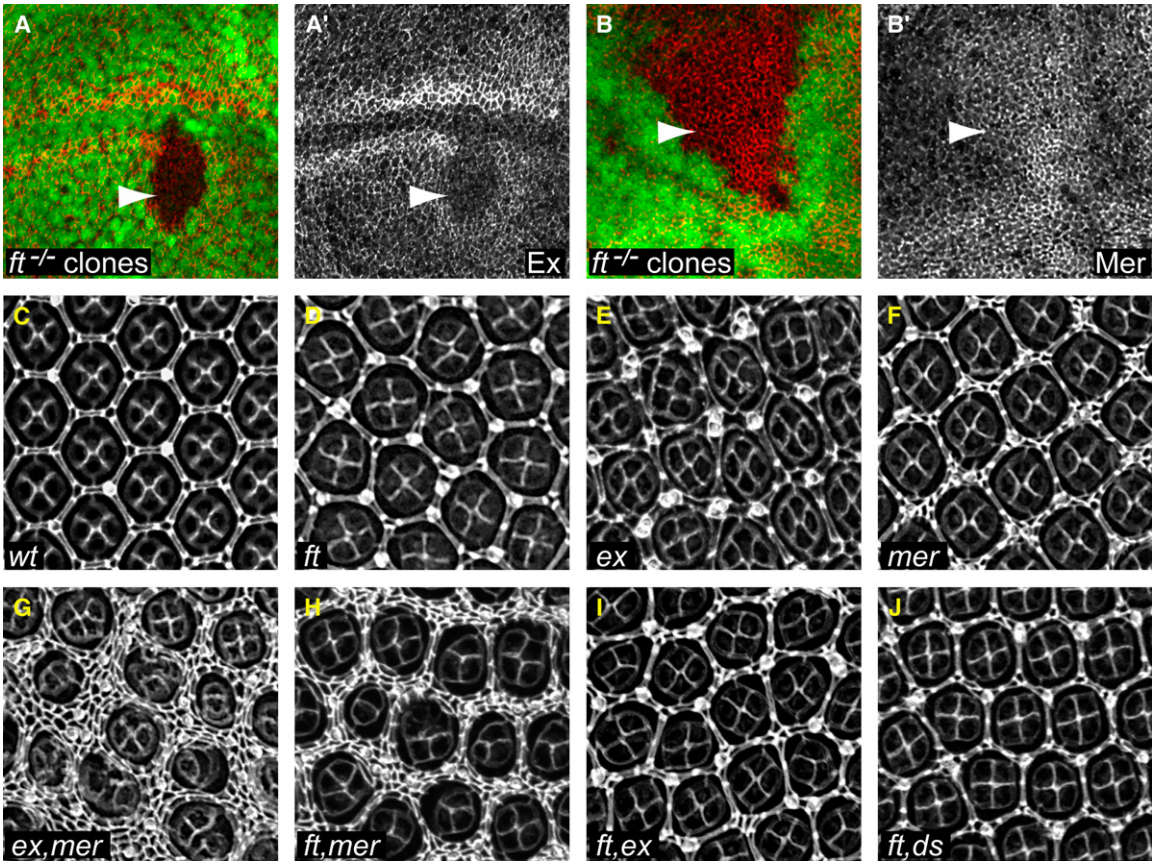


Figure 5. Fat Is Required for Expanded Membrane Localization, and Fat and Merlin Cooperate to Regulate Cell Numbers in the Retina

(A and B) *ft* mutant clones in wing imaginal discs had decreased levels of Ex but slightly increased levels of Mer proteins at the apical membranes (red in [A] and [B] and grayscale in [A′] and [B′]). Clones are marked by the absence of GFP expression (green in [A] and [B]). Alleles were $ft^{422}$ (A) and $ft^{fd}$ (B). Arrowheads point to mutant clones.

(C–J) Mid-pupal retinae stained with Discs large (Dlg) antibodies that localize to apical junctions and visualize cell outlines. (C) shows a wild-type retina. The following mutant retinae are shown: (D) $ft^{422}$, (E) $ex^{BQ}$, (F) $mer^{4}$, (G) $mer^{4}$ $ex^{e1}$, (H) $mer^{4}$ $ft^{422}$, (I) $ft^{422}$ $ex^{BQ}$, and (J) $ft^{422}$ $ds^{UA071}$. The phenotype of the $mer^{4}$ $ft^{422}$ double-mutant retina was similar to the $mer^{4}$ $ex^{e1}$ mutant retina.

(Figure S3), even though *ft* mutant cells upregulated *ex* expression (Figure S2), suggesting that Ft affects the stability of Ex as well as its localization. Strikingly, the localization of Mer was not notably affected in *ft* mutant cells (Figure 5B). Rather, the levels of Mer were slightly increased, probably as a result of the reduced levels of Hpo signaling, which normally suppresses the expression of *mer* in a negative feedback loop [5]. Our results thus indicate that Ft is specifically required for the localization and stability of Ex.

### Fat and Merlin Cooperate to Regulate Cell Number in the Retina

As noted above (Figure 1), the overgrowth phenotypes of *ft* mutant clones are very similar to those of *hpo* mutant clones. However, the *ft* mutant phenotypes are not as strong as those of complete loss of Hpo signaling. Rather, the *ft* mutant phenotypes resemble partial loss of Hpo signaling. This difference is most conspicuous in the pupal retina. Whereas loss of *hpo* or *wts* causes a massive increase in the number of interommatidial cells [5, 8], *ft* mutant retinae show only a few more pigment cells (Figures 5C and 5D). Interestingly, the pupal retina phenotypes of *ft* are very similar to those of *ex* mutants, which also reduce but do not abrogate Hpo signaling (Figure 5E, [5]). We have previously reported that Ex acts in parallel to Mer. Like *ex* mutants, *mer* mutant retinae show some extra interommatidial cells (Figure 5F), but *ex;mer* double-mutant retinae show a large excess of interommatidial cells very similar to complete loss of *hpo* signaling (Figure 5G, [5]). Because Ft is required for membrane localization of Ex but not Mer, we hypothesized that Ft may signal mainly through Ex. If true, then Ft, like Ex, may act in parallel to Mer. Indeed we found that *ft;mer* double-mutant retinae had large amounts of extra interommatidial cells comparable to *ex;mer* double mutants (Figure 5H). In contrast, *ft,ex* double-mutant retinae did not have large numbers of interommatidial cells and resembled the *ex* and *ft* single mutants (Figure 5I). We note, however, that *ft,ex* double-mutant clones had stronger overgrowth phenotypes than the single mutants in the head outside the retina, indicating that Ft and Ex may also have functions independent of each other. Nevertheless, our data support a model in which Ft signals mainly through Ex but in parallel to Mer to regulate Hpo signaling.

The hypothesis that Ft acts mainly through Ex then raised the question of whether there is another receptor

acting upstream of Mer. Because Ft acts in a partially redundant manner with Ds in growth control [32], we asked whether Ft acts together with Ds to control interommatidial cell number. Using a strong *ds* loss-of-function allele, we found that removal of Ds, together with *ft*, did not significantly enhance the extra interommatidial cell phenotype of *ft* mutant retinae (Figure 5J). Other, unknown receptors may thus act upstream of Mer.

## Discussion

Our data functionally link the growth-control function of Ft with Hpo signaling. We propose a model in which Ft is required to transduce a growth-regulatory signal to Hpo and Wts, which in turn regulate the activity of Yki to control the expression of target genes (Figure 6). Several lines of evidence place the growth-regulatory activity of Ft upstream in the Hpo signaling pathway. First, the overgrowth phenotypes of *ft* mutant cell clones resemble those of loss of Hpo signaling. This includes the overgrown imaginal discs and the overgrowths in adult structures, the growth advantage of mutant cells over wild-type cells, and the upregulation of known Hpo target genes. The combination of these phenotypes is characteristic for loss of Hpo signaling. Second, the phenotypes caused by overexpression of Ex and Hpo are epistatic to the phenotypes caused by loss of *ft*, and hyperactivation of Hpo is sufficient to rescue transcriptional defects of *ft* mutant cells. Third, Ft induces phosphorylation of Wts and regulates the activity of Yki in a cell-culture-based assay. This effect on Yki requires the function of Hpo and Wts. Fourth, Ft is required for membrane localization and stability of Ex. Fifth, Ft and Ex act in parallel to Mer to restrict interommatidial cell number. Together, our data support a model in which Ft acts upstream and through Hpo signaling to perform its growth-control function (Figure 6).

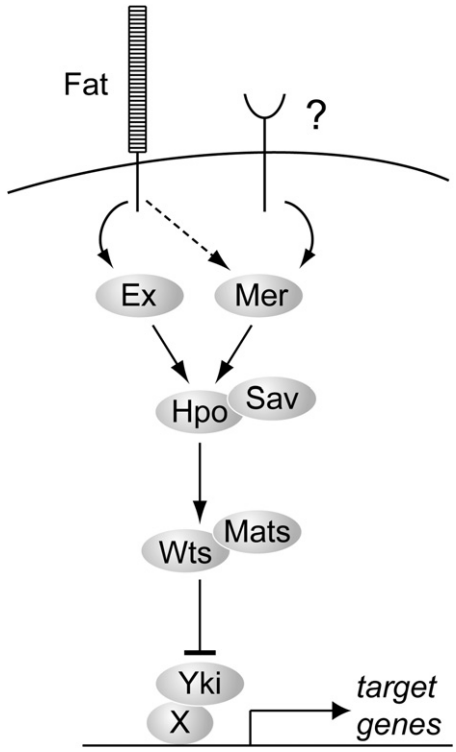


Figure 6. Model of the Hpo Signaling Pathway

Model of how Ft regulates growth. Ft acts upstream of Ex to regulate the activity of Yki through the Hpo and Wts kinases. Yki is a transcriptional coactivator and presumably associates with a transcription factor (X) to regulate the expression of target genes. Mer acts in parallel to Ex and may transduce a signal from another, unknown cell-surface receptor. It is possible that Ft also requires Mer for its growth-control function.

*ft* mutants produce overgrowth phenotypes that show all the hallmarks of *hpo* mutants. However, the phenotypes of *ft* are not as strong as those of complete loss of Hpo signaling and resemble partial loss of Hpo signaling. In fact, the *ft* mutant phenotypes very closely resemble the *ex* mutant phenotypes. We have previously reported that Ex and Mer act in parallel to regulate Hpo signaling and that *mer;ex* double mutants phenocopy complete loss of Hpo signaling, whereas the single mutants have weaker phenotypes [5]. Because *ft* mutant cells have defects in Ex but not Mer localization, and because Ft acts in parallel to Mer similarly to Ex, Ft may act mainly through Ex to regulate Hpo signaling. Other receptors may then act upstream of Mer to regulate Hpo in parallel to Ft/Ex (Figure 6). Ds does not appear to be this missing component, because *ft,ds* double mutants did not produce the large excess of interommatidial cells typical for complete loss of Hpo signaling. Finally, our data do not exclude more complicated scenarios where Ft also regulates Mer and where Ex is also regulated by other factors.

Ft is required not only for growth control but also for planar cell polarity (PCP) and for proximal-distal (P-D) patterning of the adult appendages [26–29, 32, 34, 35]. Ft mutant wings thus have abnormal hair orientation (PCP defects), are foreshortened (P-D patterning defects), and are overgrown (growth defects) [26–35]. Loss of Hpo signaling specifically affects tissue size, but *hpo*, *wts*, and *sav* mutants do not show the defects in proximal-distal patterning and in planar cell polarity observed in *ft* mutants. Mutations in *ex* show very weak polarity phenotypes in the eye, that are, however, much weaker than the phenotypes observed in *ft* mutants [40]. It thus appears that Hpo specifically mediates the action of Ft on regulating tissue size.

How does Ft regulate Ex and Hpo pathway activity? We found that *ft* mutant clones had reduced levels of Ex localized to the plasma membrane. The defects in Hpo pathway activity may thus be a consequence of the loss of Ex from the plasma membrane. In this scenario, Ft acts as a scaffold that is required to recruit Ex to the membrane, making it available for another regulator or receptor. Alternatively, Ft may act as a receptor and modulate the activity of Ex. Because neither Ex nor Ft has domains with known catalytic activities, it is currently challenging to distinguish between these two possibilities. A version of Ft that lacks the extracellular domain, FtΔECD, is sufficient to mediate the pleiotropic functions of Ft in regulating PCP and growth [32], and we found that FtΔECD can regulate Hpo signaling. The intracellular domain of Ft contains several conserved motifs, and one of them interacts with Atrophin, which may mediate the effects of Ft on planar cell polarity [36]. Ft may thus exert its diverse functions by recruiting and

interacting with members of different pathways through distinct motifs in its intracellular domain.

Does a Fat-like receptor act upstream of Hpo signaling in vertebrates? All currently known components of the Hpo signaling pathway in *Drosophila* are highly conserved in vertebrates. Given the conservation of the Hpo pathway components, it is likely that also the human homologs act together in a pathway similar to the one discovered in *Drosophila*. Indeed, the human Hpo homologs bind to hSav1 and phosphorylate and activate Lats1/2, the Wts homologs (reviewed in [4]). Vertebrates have four Ft homologs, and Ft4, the ortholog of *Drosophila* Ft, is conserved along its entire sequence including the intracellular domain [43–45]. Currently, we have no information on the function of vertebrate Ft4. Several of the vertebrate Hpo pathway components act as tumor-suppressor genes and have been implicated in regulating cell proliferation and apoptosis (reviewed in [4]). It will be interesting to find out whether Ft4 also regulates proliferation and apoptosis in vertebrates and whether it does so through the Hpo pathway. Our data that link Ft to Hpo signaling may thus have important implications for the study and treatment of neurofibromatosis and other cancers.

## Experimental Procedures

### *Drosophila* Stocks

Mutant clones were induced by using the FLP/FRT system [46, 47]. For generating *ft,ex* double-mutant, *ft,ds* double-mutant, or *ft*, *ex*, *mer*, *hpo*, or *wts* mutant clones, the following alleles were flipped against corresponding *ubiGFP*-marked *FRT* chromosomes: $ft^{422}$ [26], $ft^{G\text{-}rv}$ (null) [19, 32], $ft^{fd}$ (null) [19, 32], $ds^{UAO71}$ [33], $ex^{e1}$ (null) [18], $ex^{BQ}$ (null) [5], $mer^{4}$ (null) [48], $hpo^{42\text{-}47}$ (null) [10], and $wts^{x1}$ (null) [14]. Generation of *mer;ex* double-mutant clones was described previously [5]. *ex-lacZ* expression in *ft* mutants was assayed by using a strong hypomorphic *ft* allele, $ft^{spy}$, that we found in a genetic screen (unpublished data). Overexpression was done with the UAS-GAL4 system [49] and the following stocks: *UAS-ft* [35], *UAS-ftΔICD* [32], *UAS-ftΔECD* [32], *UAS-ex* [41], and *UAS-hpo* [8]. Other stocks were $ex^{49}$ [5], $ex^{697}$ [18], *DIAP1-lacZ* [50], *CycE-lacZ* (16.4 construct [51]), *fj-lacZ* [52], $wts^{P2}$ [13], and $yki^{B5}$ [16].

### Scanning Electron Microscopy and Immunohistochemistry

Scanning electron microscopy (SEM) of adult flies was done following the HMDS method [7]. Antibody stainings of imaginal discs were done as described [7]. The following antibodies were used (source and dilutions in parentheses): mouse α-Dlg (DSHB, 1:300), guinea-pig α-Mer (R. Fehon, 1:4000), guinea-pig α-Ex (R. Fehon, 1:2000), rabbit α-Ex (A. Laughon, 1:1500), rat-α-Ft (M.A. Simon, 1:2000), mouse α-BrdU (Becton-Dickinson, 1:50), mouse α-DIAP-1 (B. Hay, 1:200), mouse α-CycE (H. Richardson, 1:40), and mouse α-βGal (Promega, 1:2000). Secondary antibodies were donkey Fab fragments from Jackson ImmunoResearch. BrdU incorporation was done as described by incorporating BrdU for 1 hr [7].

### Cell Culture and Western Blotting

The Ex and Mer constructs were described in [5]. For Ft expression, the Ft coding region was excised from a pUAST-Ft construct [35] by NotI-KpnI digestion and inserted into the pAc5.1 vector (Invitrogen). The FtΔECD construct was generated by PCR and consists of the Ft signal peptide fused to a V5 tag followed by the Ft coding sequence from amino acid 4550 to the stop codon cloned in the pAc5.1 vector. All other constructs were gifts from Duojia Pan [10, 16]. *Drosophila* S2 cells, cultured in Schneider's medium containing 10% fetal bovine serum (FBS) and antibiotics, were transiently transfected by using Cellfectin (Invitrogen) and collected 48 hr after transfection. Cells were lysed in SDS sample buffer, and western blots were performed according to standard protocols. Antibodies used were α-V5 (Invitrogen), α-αTub (Sigma, St. Louis, Missouri), and α-Ex (R. Fehon). For phosphatase treatments, cells were lysed in IP buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 0.5% NP40, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin) and V5-Wts proteins were immunoprecipitated. Beads were washed three times in phosphatase treatment buffer (50 mM Tris-HCl [pH 7.5], 0.1 mM EDTA, 0.5 mM MgCl2, 0.5 mM MnCl2, 0.5 mM CaCl2), and 0.3 units of PP2A (Upstate, Charlottesville, Virginia) were added in a total volume of 75 μl, followed by 30 min incubation at 37°C. The reaction was terminated by addition of an equal volume of SDS sample buffer.

Luciferase reporter-gene assays were performed by transfecting 10 ng of Yki-Gal4 or Gal4-FL plasmids with 0.5 ng of *UAS-luciferase* plasmid in triplicates with or without plasmids expressing Ex, Mer, Hpo, Sav, Wts, Ft, and FtΔECD in 48-well plates. Luciferase assays were performed with the Dual Luciferase Reporter Assay System (Promega) and a $20/20^{n}$ Luminometry System with Single Auto-Injector (Promega) 96 hr after transfection.

For protein knockdown, dsRNAs were synthesized with the MEGAscript RNAi kit (Ambion) from PCR products containing the T7 promoter (TAATACGACTCACTATAGGG). Primer pairs were as follows: *lacZ* forward, 5′-TAATACGACTCACTATAGGGGTTTGTTACTCGCTCACATT-3′; *lacZ* reverse, 5′-TAATACGACTCACTATAGGGTCGAATCAGCAACGGCTTGC-3′; *wts* forward, 5′-TAATACGACTCACTATAGGGAAGGCGGCCACGGTG-3′; *wts* reverse, 5′-TAATACGACTCACTATAGGGCTCCTTCTCCTTGGAGATCT-3′; *hpo* forward, 5′-TAATACGACTCACTATAGGGCTGTGTGGCAGACATATGGT-3′; *hpo* reverse, 5′-TAATACGACTCACTATAGGGCTCATCCACACCTTGCTCT-3′; *ex* forward, 5′-TAATACGACTCACTATAGGGGAGAAGAAACGGAGAGTGTAT-3′; and *ex* reverse, 5′-TAATACGACTCACTATAGGGGGCCACAGAGACCAGTTTT-3′.

Cells were incubated with 3.5 μg dsRNA/well (48-well plates) in 125 μl Schneider's medium. After 1 hr, 125 μl Schneider's medium containing 20% FBS was added to each well.

### Supplemental Data

Supplemental Data include three figures and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/21/2090/DC1/.

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