The tumor suppressor genes *dachsous* and *fat* modulate different signalling pathways by regulating *dally* and *dally-like*

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The activity of different signaling pathways must be precisely regulated during development to define the final size and pattern of an organ. The *Drosophila* tumor suppressor genes *dachsous* (*ds*) and *fat* (*ft*) modulate organ size and pattern formation during imaginal disc development. Recent studies have proposed that Fat acts through the conserved Hippo signaling pathway to repress the expression of *cycE*, *bantam*, and *diap-1*. However, the combined ectopic expression of all of these target genes does not account for the hyperplasic phenotypes and patterning defects displayed by Hippo pathway mutants. Here, we identify the glypicans *dally* and *dally-like* as two target genes for both *ft* and *ds* acting via the Hippo pathway. Dally and Dally-like modulate organ growth and patterning by regulating the diffusion and efficiency of signaling of several morphogens such as Decapentaplegic, Hedgehog, and Wingless. Our findings therefore provide significant insights into the mechanisms by which mutations in the Hippo pathway genes can simultaneously alter the activity of several signaling pathways, compromising the control of growth and pattern formation.

Drosophila | glypicans | Hippo pathway | protocadherin

The final size and pattern of organs depend on the fine control of cell proliferation and differentiation during development. It is a widespread assumption that this control is mediated by the coordinated activity of multiple signaling pathways. However, little is known about the genetic mechanisms by which the activity of several pathways is simultaneously modulated and about their deregulation in tumoral situations. Mutations in the *Drosophila* protocadherin genes *fat* (*ft*) and *dachsous* (*ds*) are associated with alterations in the mechanisms of control of cell proliferation, establishment of planar cell polarity (PCP) (1, 2), orientation of cell division (3), proximo–distal patterning of appendages (2, 4), and pattern formation (4, 5). Interestingly, some of these defects resemble those caused by mutations in the conserved transduction cascades of Wnt, TGF-β, and Hedgehog, suggesting that the activity of these signaling pathways may be regulated by *ft* and *ds*. Therefore, the detailed analysis of these mutants emerges as an appropriate model to explore the mechanisms by which the activity of different pathways can be simultaneously controlled and their influence in the definition of the size and pattern of an organ studied.

Fat has been proposed to be a receptor of the Hippo pathway (6–8), acting as an upstream component of the signaling pathway together with the FERM domain proteins Expanded (Ex) and Merlin (Mer) (9). The activation of the pathway in normal conditions leads to a sequential cascade of phosphorylations of a core of protein kinases [Hippo (Hpo), Salvador (Sav), and Warts (Wts)], which prevents the nuclear translocation of the transcriptional coactivator Yorkie (Yki) (7, 8). By contrast, in misregulated situations, Yki is accumulated into the nucleus (10, 11), favoring the expression of proliferation-promoting genes such as *bantam*, *cyclin E*, and the cell death inhibitor *diap-1* (7, 8). Importantly, the ectopic expression of these three genes alone is not sufficient to account for the hyperplastic phenotypes and patterning defects observed in Hippo pathway mutant cells (12, 13), suggesting that other key target genes remain unidentified. In addition, the relationship of the Hippo pathway with Ds, the putative ligand of Fat, is still unclear (7).

In this work we explore the activity of multiple signaling pathways in *ft* and Hippo pathway mutants and their contribution to the hyperplastic phenotypes and patterning defects displayed by these mutants. In addition, we investigate the role of *ds* in the context of Hippo signaling. Our results provide evidence that the *Drosophila* conserved heparan sulfate proteoglycans (HSPGs), *division abnormally delayed* (*dally*) and *Dally-like protein* (*dlp*), are two targets of *ds* and *ft* acting through the Hippo signaling pathway. We show that these glypicans are negatively regulated by *ds* and *ft* and that this effect is mediated by the transcriptional coactivator Yki. Dally and Dlp are cell surface proteins that adjust the extracellular distribution and signal efficiency of secreted ligands such as Wingless (Wg), Hedgehog (Hh) and Decapentaplegic (Dpp) (14–16). Therefore, we propose a molecular mechanism by which the activity of several signaling pathways can be simultaneously compromised in Hippo pathway mutant cells.

Results

***ds* and *ft* Modulate the Expression of Target Genes of the Hh, Wg, and Dpp Signaling Pathways.** Mutations in *ds* and *ft* alter the growth, shape, and patterning of different organs in *Drosophila*, including wings, legs, and eyes (4–6). Some of these defects have been explained by the requirement of these genes in the modulation of Wg signaling (4, 5). Other phenotypes, such as the loss of cross-veins [compare supporting information (SI) Fig. S1 *A* and *B*] and the misplacement or presence of ectopic veins (Fig. S1*B*), resemble those caused by changes in the activity of Dpp or Hh signaling. We examined the effect of the loss of *ds* and *ft* on the activity of different signaling pathways by analyzing the expression of target genes of Dpp, Hh, and Wg signaling during wing disc development. In ds^{D36} clones, some cells autonomously reduce the levels of a phosphorylated form of Mad (pMad), a direct readout of Dpp signaling (yellow arrowhead in Fig. 1 *A* and *A′*). However, other mutant cells, especially those adjacent to the surrounding wild-type cells, increase the levels of this protein, thus broadening the domain of pMad expression (red arrow-

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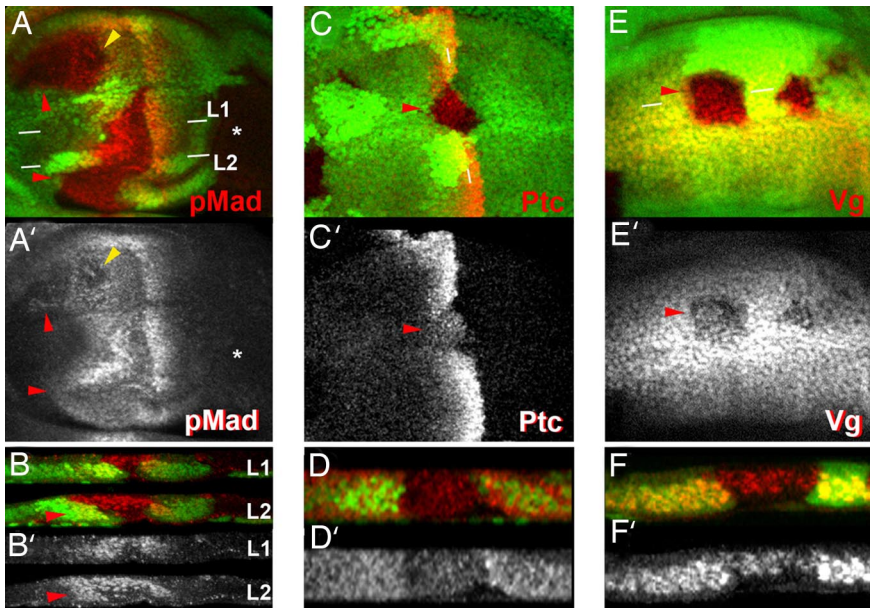

Fig. 1. Ds modulates the activity of different signaling pathways. (*A–F*) Third-instar wing discs containing clones of a null allele of *ds* (ds^{D36}). Mutant cells are marked by the absence of GFP (in green). (*A* and *B*) Whereas some ds^{D36} cells can express pMad (in red) beyond its normal domain of expression (red arrowheads in *A* and *A′*), other cells show a reduction in pMad levels (yellow arrowheads in *A* and *A′*). ds^{D36} clones located in the lateralmost part of the disc do not up-regulate pMad (asterisk). (*B* and *B′*) Two different optical Z sections shown in *A* (L1 and L2) illustrate the distribution of pMad. This protein is nonautonomous up-regulated in some of the surrounding wild-type cells (red arrowheads in *B* and *B′*). (*C* and *D*) The expression of Ptc (in red) and (*E* and *F*) Vg (in red) is reduced in ds^{D36} cells (red arrowheads). (*D* and *F*) Ptc and Vg expression, respectively, Z sections along A/P axis in ds^{D36} clones. Gray, expression of pMad (*A′* and *B′*), Ptc (*C′* and *D′*), and Vg (*E′* and *F′*).

heads in Fig. 1 *A* and *A′*). We never find ectopic expression of pMad in mutant clones at lateral regions of the wing disc. By contrast, we occasionally observe that the expression of pMad is nonautonomously increased in the surrounding wild-type cells (red arrowhead in Fig. 1 *B* and *B′*, L2). Moreover, the expression of target genes for Hh and Wg pathways, such as *patched* (*ptc*) (Fig. 1 *C* and *C′*, *D* and *D′*) and *vestigial* (*Vg*) (Fig. 1 *E* and *E′*, *F* and *F′*), is slightly reduced in ds^{D36} clones.

In mutant cells for ft^{422} and ft^{G-rv} alleles, the expression of Ptc and Vg (Fig. S1) can be also reduced, although the effects are weaker than those observed in *ds* mutant alleles. We do not find changes in the expression of pMad in these mutant cells (data not shown). We obtained similar results for the hypomorphic ds^{UAO71} allele, suggesting that the different behavior of *ds* and *ft* alleles could be caused by the hypomorphic condition of the *ft* alleles. Together, these findings suggest a model in which both Ds and Ft regulate the activity of these pathways, although we find differences in the strength of this ability.

Apico–basal cell polarity is essential for epithelial integrity and therefore to ensure the mechanisms of signaling. Because Ds and Ft accumulate at the subapical region of the cell membrane (data not shown), their loss of function could affect the apico–basal cell polarity and indirectly compromise the activity of different signaling pathways. However, in ds^{D36} or ft^{422} cells, the localization of the apical protein Armadillo (Arm), the baso–lateral protein Disc large (Dlg), and the basal protein Laminin (Lan) is normal (data not shown), suggesting that the signaling defects observed in *ds* and *ft* mutant cells are not caused by major changes in the integrity of apico–basal cell polarity.

***ds* and *ft* Regulate the Transcription of *dally* and *dlp*.** A common feature of the Dpp, Hh, and Wg pathways is that they function in a concentration-dependent manner and are activated by secreted ligands. A simple explanation of our results might be that *ds* and *ft* affect the diffusion and/or reception of these molecules. The glypicans, Dally and Dlp, are cell surface proteins that play a key role in the distribution and activity of secreted ligands (14–16). When the functions of *dally* or *dlp* are compromised, the activity of different signaling pathways such as Dpp, Hh, or Wg is altered (14–16). Interestingly, both ds^{D36} and ft^{422} mutant cells autonomously accumulate high levels of Dlp protein in the wing disc (Fig. 2 *A* and *A′* and Fig. S2 *A* and *A′*) as well as in other imaginal discs (Fig. S2 *C* and *C′*, *D* and *D′*). Moreover, *dlp* mRNA (Fig. 2 *C* and *C′* and Fig. S2 *E* and *E′*) is autonomously up-regulated in all examined ds^{D36} and ft^{422} mutant clones. Because the functions of *dlp* and the other *Drosophila* proteoglycan *dally* are partially redundant (17), we also examined whether *dally* expression was modified in ds^{D36} and ft^{422} clones. Indeed, the levels of *dally-LacZ* expression in these mutant cells are higher than in surrounding wild-type cells (Fig. 2 *B* and *B′* and Fig. S2 *B* and *B′*). This up-regulation was not observed in hypomorphic *ds*/*ft* alleles such as ds^{UAO71} and ft^{18} (data not shown). Together, our results suggest that *ds*/*ft* signaling provides a general mechanism to regulate negatively the transcription of the glypicans *dally* and *dlp*.

***ds* and *ft* Regulate the Transcription of *dally* and *dlp* via the Hippo Signaling Pathway.** There are at least two signaling branches downstream of *ft* signaling: the PCP branch, which requires the transcriptional corepressor Atrophin (Atro) (18), and the tumor suppressor branch, which restricts cell proliferation via the Hippo signaling pathway (7, 8). In clones for the null allele of $atro^{35}$ we cannot detect changes in either the abundance or localization of Dlp (Fig. S2 *F* and *F′*), indicating that the PCP branch is not required for this up-regulation. Conversely, when the Hippo signaling pathway is compromised, either by inducing wts^{X1} mutant clones (Fig. S2*G*) or by overexpressing *yki* (Fig. 2 *D* and *D′* and Fig. S2 *H* and *H′*), the levels of Dlp are increased in all mutant cells. These data are consistent with a model in which *ds* and *ft* repress the expression of *dally* and *dlp* via the Hippo signaling pathway. Supporting this model, the expression

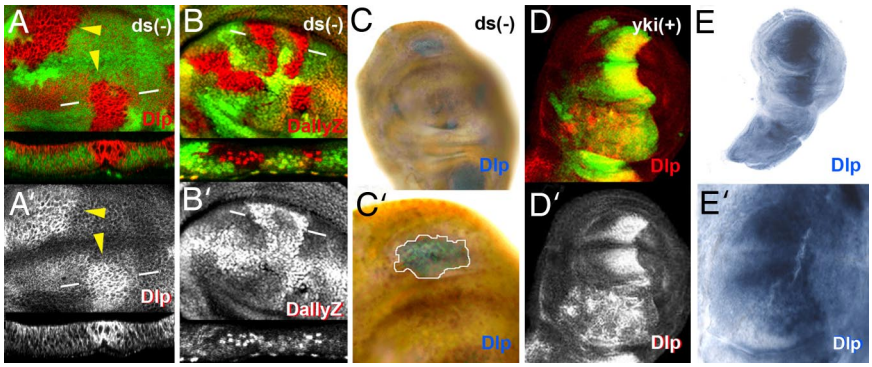

Fig. 2. *dally* and *dlp* are transcriptionally up-regulated in *ds* mutant cells. (*A*–*C*) Third-instar imaginal wing discs containing ds^{D36} mutant clones. (*D* and *E*) Third-instar wing discs expressing *dpp-Gal4*/UAS-*yki*. (*A* and *A′*) ds^{D36} cells, marked by the absence of GFP (in green) express high levels of Dlp protein (red) (yellow arrowheads) and (*B* and *B′*) *dallyZ* in a cell-autonomous manner. Optical Z-sections from *A* and *B* are shown. White bar indicates the position of the section. (*C* and *C′*) The levels of *dlp* mRNA (blue) are increased in ds^{D36} cells, marked by the absence of β-galactosidase staining (brown). (*C′*) High magnification of a clone (outlined in white) shown in C. (*D* and *D′*) Third-instar wing discs expressing *dpp-Gal4*/UAS-*yki* show up-regulation of Dlp protein (in red) and (*E* and *E′*) *dlp* mRNA (blue). Gray, expression of Dlp protein (*A′* and *D′*) and *dallyZ* (*B′*).

of *dlp* mRNA (Fig. 2 *E* and *E′*) is also increased in clones of *yki*-expressing cells.

It has been reported that the ectopic expression of *dlp* causes autonomous accumulation of Wg in the cell surface (16). Because *yki*-expressing cells display high levels of Dlp, we expect to find a similar effect in this mutant condition. Consistently, we observe accumulation of the pool of extracellular Wg in posterior cells of wing discs expressing *yki* under the control of *en-Gal4* (Fig. S2 *I* and *I′*).

Our results suggest that *dlp* and *dally* are two targets of the Hippo signaling pathway. Therefore, a reduction of the activity of this pathway should decrease the expression of these glypicans. Because the poor viability and small size of *yki* mutant clones make their analysis very difficult (data not shown), we have blocked the activity of Yki overexpressing a wild-type form of Hpo (19) under the control of *sal-Gal4*. These mutant wings are extremely reduced in size (Fig. S3*A*), and they display high levels of apoptosis (as revealed by anti-caspase 3 staining (Fig. S3 *B* and *B′*). Coexpression of the antiapoptotic factor P35 strongly suppresses the wing reduction phenotype (Fig. S3*C*) and the cell death effect (Fig. S3 *D* and *D′*). Consistent with our results, in *sal-Gal4*;UAS-*P35*/UAS-*Hpo* wing discs, Dlp expression is reduced and shows altered localization (Fig. 3 *A* and *A′*). By contrast, *engrailed* (*en*) expression is normal, thus indicating that these cells are healthy and their gene transcription is not globally affected (Fig. S3*E*). Together, our results are consistent with a model in which the expression of *dally* and *dlp* is regulated by *ft* and *ds* acting through the Hippo signaling pathway. According to this model, activation of the Hippo pathway in *ft* or *ds* mutant cells should prevent Dlp up-regulation. Indeed, we cannot detect the up-regulation of Dlp found in *ft* mutant clones (Fig. 3 *B* and *B′*) in *ft* mutant cells that ectopically express Hpo (Fig. 3 *C* and *C′*).

Co-overexpression of *dally* and *dlp* Phenocopies Some of the Effects Caused by the Loss of Function of *ds*/*ft* and Overexpression of *yki*. The ectopic expression of Dally and Dlp should mimic some of the effects observed in either *ds*/*ft* mutant clones or *yki*-expressing cells. To test this hypothesis, we analyzed the phenotypes caused by overexpression of UAS-*dally* and UAS-*dlp*, alone or in combination, under the control of *ap-Gal4*. Ectopic expression of UAS-*dally* expands the range of Dpp signaling in the dorsal compartment of the wing disc, as indicated the broadened domain of pMad expression in this compartment (Fig. S4 *A* and *A′*). By contrast, overexpression of UAS-*dlp* leads to reduction of pMad expression (Fig. S4 *B* and *B′*). The activity of Hh signaling is also altered in these mutant conditions, as revealed by Ptc expression. Thus, whereas the band of Ptc expression in the dorsal compartment of the *ap-Gal4*/UAS-*dally* wing disc is slightly broadened, it is narrower in *ap-Gal4*/UAS-*dlp* (Fig. S4 *A″* and *B″*). Coexpression of both genes in *ap-Gal4*/UAS-*dally*; UAS-*dlp* wing discs results in a combined effect. Hence, the pMad domain is expanded close to the D/V border (Fig. 4 *A* and *A′*, arrow), but it is also narrowed or down-regulated in small patches of cells (arrowhead in Fig. 4*A′*). In these discs, the stripe of Ptc expression is thinner in the dorsal compartment (Fig. 4*A″*). This result can be explained considering the opposing effects of *dally* and *dlp* on the regulation of the activity of different signaling pathways (16, 17). The up-regulation of *dally* and *dlp* in *yki*-expressing cells (Fig. 2) suggests that the activity of Dpp, Hh, and Wg signaling could be also affected in this genetic back-

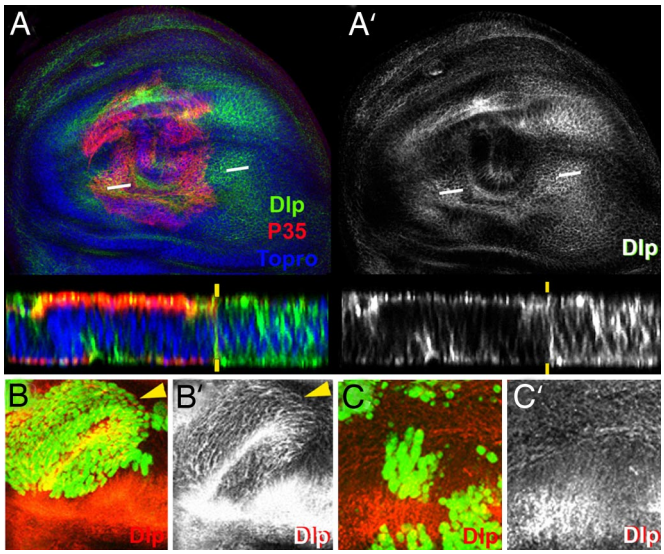

Fig. 3. Activation of the Hippo pathway causes down-regulation of Dlp. (*A* and *A′*) In third-instar *sal-Gal4*/UAS-*P35*; UAS-*HpoWT* wing discs, the expression of Dlp (green) is reduced within the *sal-Gal4* domain (marked in red with anti-P35). Z sections are shown to visualize Dlp levels (in green). White bars indicate the position of the section. Nuclei were monitored with Tropo staining (blue). (*B* and *B′*) In ft^{G-rv} clones, positively marked with GFP (green), the levels of expression of Dlp (red) (yellow arrowhead) are higher than in the wild-type surrounding cells. (*C* and *C′*) Ectopic expression of UAS-*HpoWt* in ft^{G-rv} cells (green) prevents the accumulation of high levels of Dlp (red).

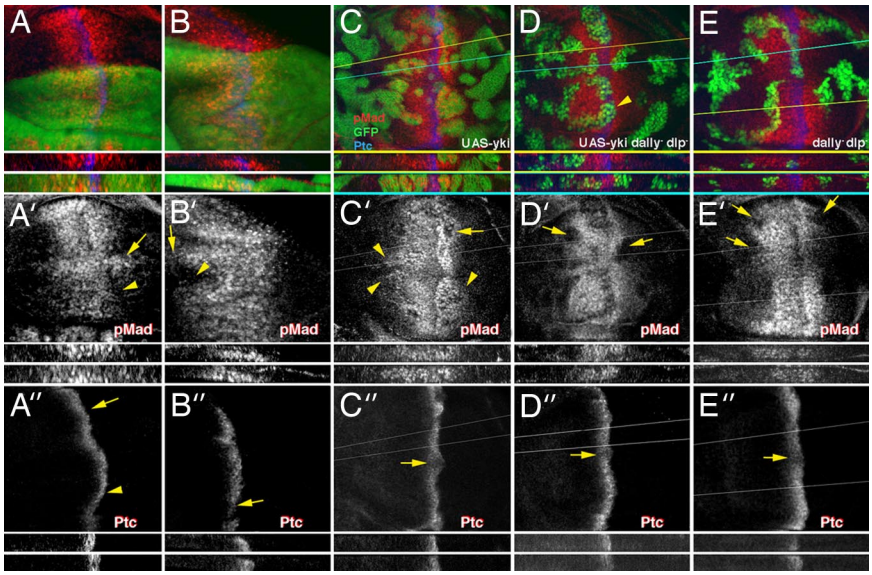

Fig. 4. Ectopic expression of *yki* mimics the signaling defects caused by overexpression of *dally* and *dlp*. (*A–E*) Analysis of pMad and Ptc expression in third-instar wing discs of the following genotypes: *ap-Gal4*/UAS-*dally*; UAS-*dlp* (*A–A″*), *ap-Gal4*/*UAS-yki* (*B–B″*), and discs containing cell-expressing clones of UAS-*yki* (*C–C″*), UAS-*yki*; *FRT2A dally*^{80} *dlp*^{A187} (UAS-*yki dally*$^{-}$*dlp*$^{-}$) (*D–D″*), and *FRT2A dally*^{80} *dlp*^{A187} (*E–E″*). In all wing discs, the cells overexpressing different transgenes were marked with GFP in green and stained with anti-pMad (red) and anti-Ptc (blue). (*A*) Co-overexpression of UAS-*dally*; UAS-*dlp* produces a combination of phenotype; close to the D/V border the expression of pMad is expanded (yellow arrow in *A′*), whereas in other dorsal regions pMad is down-regulated (arrowhead in *A′*). (*A″*) In dorsal cells, the stripe of Ptc expression seems thinner (yellow arrowhead) than in ventral cells (yellow arrow). (*B* and *B′*) In *ap-Gal4*/*UAS-yki* wing discs, pMad expression is expanded in some dorsal cells (yellow arrow), whereas it is down-regulated in other dorsal cells (yellow arrowhead). (*B″*) In dorsal cells, the stripe of Ptc seems thinner (yellow arrow). Below each panel, two optical Z sections (from ventral and dorsal compartments, respectively) illustrate the distribution of pMad and Ptc along A/P axis. Z sections were aligned with respect to Ptc expression. (*C* and *C′*) Some clones of *yki*-expressing cells express pMad outside the normal pMad expression domain (yellow arrowheads in *C′*), whereas other clones display reduced pMad levels (yellow arrow in *C′*). (*C″*) The levels of Ptc are down-regulated in some of these clones (yellow arrow). (*D* and *D′*) Clones of UAS-*yki*; *dally*$^{-}$*dlp*$^{-}$ cells are unable to up-regulate pMad (arrowhead in *D* and arrows in *D′*). In these clones the defect in Ptc expression caused by ectopic *yki* expression is restored. Occasionally, we find a slight down-regulation of Ptc (arrow in *D″*). (*E* and *E′*) In *dally*$^{-}$*dlp*$^{-}$ clones we always observed a reduction of the levels of pMad (red) (arrows in *E′*) and a slight reduction in Ptc expression (arrow in *E″*). Clones were induced at 84 ± 12 h after egg laying (AEL) and analyzed 72 h after induction except in *E*, where they were induced at 60 ± 12 h AEL. Two optical Z sections at different positions within the wing disc (yellow and blue lines) are shown. These sections were aligned with respect to Ptc expression.

ground. Accordingly, the ectopic expression of *yki* reproduces most of the effects caused by the coexpression of *dally* and *dlp*. Third-instar imaginal wing discs overexpressing UAS-*yki* under the control of *ap-Gal4* display large overgrowths in the dorsal compartment in contrast to the strongly reduced ventral compartment (Fig. 4 *B* and *B″*). For our analysis we have used young third-instar larvae because mature discs are completely deformed. Similar to the co-overexpression of *dally* and *dlp*, we found that the overexpression of *yki* causes an expansion of the pMad domain in some dorsal cells (arrow in Fig. 4*B′*), whereas other cells show reduced expression (arrowhead in Fig. 4*B′*). In these discs, Ptc expression along the A/P boundary looks very irregular and appears to be reduced in some cells (arrow in Fig. 4*B″*). Similar results are obtained with clones of *yki*-expressing cells (Fig. 4 *C* and *C′*). The expression of Vestigial (Vg) is down-regulated in *dpp-Gal4* UAS-*yki* wing discs, indicating that Wg signaling is also affected (Fig. S4 *C* and *C′*). All these effects are partially suppressed when *dally* and *dlp* functions are reduced. Thus, in UAS-*yki*; FRT2A *dally*^{80} *dlp*^{A187} clones the expansion of the pMad domain is abolished, and pMad expression is down-regulated (arrows in Fig. 4*D′*), as observed in FRT2A *dally*^{80} *dlp*^{A187} cells (arrows in Fig. 4*E′*). These results indicate that the effect of the Hippo pathway on the multiple signaling pathways requires up-regulation of *dlp* and *dally*.

The Overgrowth Phenotype Caused by the Loss of Function of the *ds*, *ft*, and Hippo Pathway Is Partially Suppressed by Mutant Alleles of *dally* and *dlp*. It has been proposed that *dally* and *dlp* control organ size by modulating the activity of different signaling pathways (20, 21). To test this idea, we analyzed their contribution to the hyperplastic phenotypes caused by Hippo pathway mutant cells. Because *dally* and *dlp* are negatively regulated by Hippo signaling, *dally*/*dlp* mutation should rescue the extra-growth phenotypes displayed by mutant alleles of *ds*/*ft* or Hippo pathway members. Homozygous double mutant for the loss of function alleles *dally*^{80} and *dlp*^{A187} are embryonic lethal (15). However, flies carrying a combination of both alleles over a hypomorphic allele of *dally* (*dally*^{80} *dlp*^{A187}/*dally*^{P1}) are viable and display a strong phenotype consisting of reduced wings with a truncated vein 5 (Fig. S5*B*). Wings derived from the viable combination *ds*^{D36}/*ds*^{38k} are on average 12% (n = 10) larger than control wings (Fig. S5*C*). In addition, these wings display PCP phenotypes, have a rounded shape, and lack cross-veins. Interestingly, the size and patterning defects resemble those of wings that ectopically expressed *yki* at low levels during the third instar (Fig. S5*E*). The overgrowth phenotype of *ds* mutant wings is dramatically suppressed in *ds*^{D36}/*ds*^{38k}; *dally*^{80} *dlp*^{A187}/*dally*^{P1} wings (Fig. S5*D*), but the PCP and shape defects (10) are still present (Fig. S5*F*). Similarly, a reduction in *dally* and *dlp* function significantly rescues the overgrowth phenotype observed in *ft*$^{G-rv}$/*ft*^{18} wing discs (Fig. 5*A*). Most of *ft*$^{G-rv}$/*ft*^{18} animals die during pupal stages, although some of them develop as adult pharates. These flies never hatch from the pupal case and display folded thorax and wings that do not evaginate, likely because of their large size (Fig. 5*B*). Reduction of *dally* and *dlp* function significantly rescues this extragrowth phenotype. Indeed, *ft*$^{G-rv}$/*ft*^{18}; *dally*^{80} *dlp*^{A187}/*dally*^{P1} adult pharates show evaginated wings and smaller structures than *ft*$^{G-rv}$/*ft*^{18} animals (compare wing disc and notum sizes in Fig. 5*B*). Next, we asked whether the overgrowth phenotype caused by the ectopic expression of *yki* might be also suppressed by reducing *dally* and *dlp*. Using mosaic

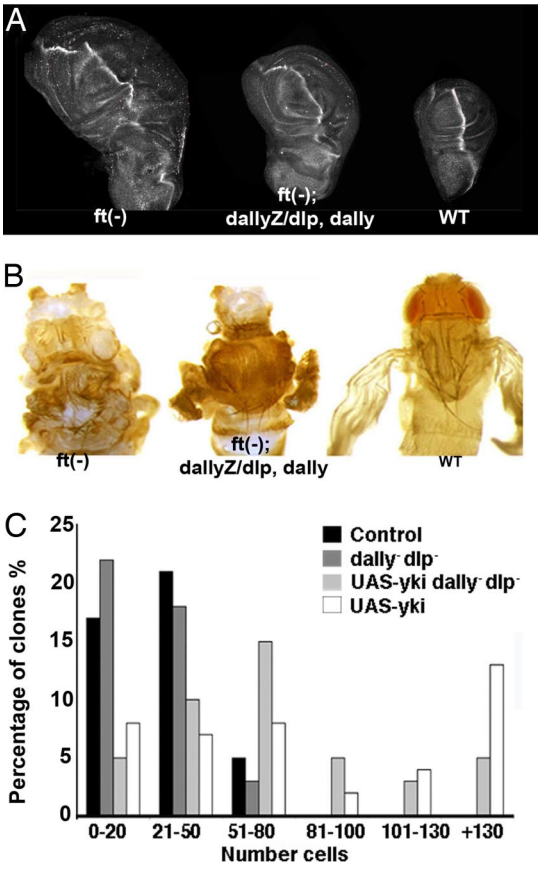

Fig. 5. *dlp* and *dally* contribute to the overgrowth phenotypes displayed by *ft* mutants and the ectopic expression of *yki*. (*A*) The size of $ft^{18}/ft^{G\text{-}rv}$; $dally^{80}$ $dlp^{A187}/dally^{P1}$ wing discs is much smaller than of $ft^{18}/ft^{G\text{-}rv}$ discs. Third-instar wing discs were stained with Ptc antibody. (*B*) Similarly, adult pharates from $ft^{18}/ft^{G\text{-}rv}$; $dally^{80}$ $dlp^{A187}/dally^{P1}$ flies show a suppression of the overgrowth phenotype compared with $ft^{18}/ft^{G\text{-}rv}$ flies. Moreover, $ft^{18}/ft^{G\text{-}rv}$; $dally^{80}$ $dlp^{A187}/dally^{P1}$ flies display everted wings, and the notum size is similar to wild type. Magnification is the same in all panels. (*C*) Quantitative analysis of the clone sizes in the different genetic backgrounds. Clones were induced at 72–96 h AEL and analyzed 72 h later ($n = 43$).

analysis (MARCM), we expressed UAS-*yki* in clones that were simultaneously mutant for *dally* and *dlp*. Clones of *yki*-expressing cells induced at 60 h AEL give rise to larvae with outsized wing discs that contain large outgrowths (Fig. S5*G*). In contrast, wing discs with UAS-*yki*; FRT2A $dally^{80}$ dlp^{A187} clones were larger than control discs but much smaller than discs containing clones of UAS-*yki* (Fig. S5*G*). The large size of the outgrowths obtained in these mutant conditions, makes it difficult to count the number of cells in each clone. To quantify the differences in size, we generated smaller clones, inducing them at 84 h AEL. They were analyzed 72 h after induction. The size of most of the control clones (UAS-*GFP*) is between 1 and 50 cells (89% of all clones analyzed), and we never find control clones larger than 80 cells (Fig. 5*C*). Although FRT2A $dally^{80}$ dlp^{A187} clones can reach the size of wild-type clones (1–50 cells), they often fall into the 1- to 20-cell category (51%). Consistent with our previous results, we find that clones of *yki*-expressing cells are much larger than control clones. We frequently (30%) observed clones with >130 cells. In UAS-*yki*; FRT2A $dally^{80}$ dlp^{A187} clones, the excess of cell proliferation caused by the ectopic expression of *yki* is partially suppressed: only 11% of these clones are larger than 130 cells, although they are still bigger than control clones (63% of clones have >50 cells compared with 11% in control clones). These findings strongly support the hypothesis that the up-regulation of *dally* and *dlp* is mediating the overgrowth caused by *ft*/*ds* and Hippo mutant cells. However, because the ectopic coexpression of *dally* and *dlp* does not induce the extragrowth observed in UAS-*yki* clones (see above), the function of *dally* and *dlp* in the modulation of growth is likely to be only permissive.

The Hippo pathway activates the expression of *cycE*, *diap-1*, and *bantam*. Thus, the overgrowth phenotype displayed by mutants in members of this pathway has been explained by the up-regulation of these factors. However, the overexpression of all these genes simultaneously does not reproduce the hyperplasic phenotypes observed in *ft* or Hippo mutants (12). Our results indicate that *yki* activates *cycE* and *diap-1* in parallel to *dally* and *dlp* because the ectopic coexpression of *dally* and *dlp* does not induce expression of these genes (data not shown). Therefore, the phenotype caused by the ectopic expression of *yki* might be due to deregulation of *cycE*, *diap-1*, *dally*, and *dlp* simultaneously. To test this hypothesis, we overexpressed *cycE*, *P35*, *dally*, and *dlp* in the wing disc under the control of *ap-Gal4*. These wing discs are never as big as *ap-Gal4*/UAS-*yki* discs, and their size is very similar to that of *ap-Gal4*/UAS-*cycE*, UAS-*P35* discs, although some specific regions (such as the hinge) seem bigger (Fig. S5 *H* and *I*). Interestingly, in *ap-Gal4*/UAS-*cycE*, UAS-*P35*, UAS-*dally*, UAS-*dlp* wing discs, we find high levels of Wg throughout the dorsal compartment, including the hinge region (Fig. S5*I*). Although, whereas the accumulation of Wg can induce overgrowth in the hinge region, it has been reported that it may have the opposite effects in the wing blade (22, 23). This result supports the idea that Hippo signaling may be modulating growth through *dally* and *dlp* in a context-dependent manner, which will depend on the specific role of each signaling pathway in each developmental context.

Discussion

The final pattern and size of an organ are determined by the activation of specific programs of gene expression in response to different signaling pathways. How the activity of these pathways is coordinated during development is a key aspect of organ size control and patterning. Recent reports have shown that the Hippo signaling pathway can control tissue growth and patterning formation (7, 8). However, the coexpression of reported target genes for the pathway (CycE, Diap-1, and *bantam*) fails to reproduce the hyperplastic phenotypes and patterning defects display by Hippo pathway mutants (12), suggesting that other unknown factors are involved. We have identified *dally* and *dlp* as two such factors, which are transcriptionally repressed by the Hippo pathway during normal development. Thus, either the up-regulation of *yki* or mutations in members of Hippo pathway (*ft*, *wts*) promote their expression. In addition, we observe the same results using mutations of *ds*, suggesting the link between this gene and the Hippo signaling pathway.

It is well described that diffusible ligands such as Dpp, Hh, and Wg play an essential role in the control of cell proliferation and patterning during development. Importantly, the HSPGs, *dally* and *dlp*, are required for the appropriate propagation and activity of these ligands (14–16, 24). Therefore, the up-regulation of HSPGs *dally* and *dlp* in Hippo pathway mutants provides a molecular mechanism to explain the simultaneous deregulation of multiple signaling pathways observed in these mutants. It also opens the possibility that other signaling pathways, activated by diffusible ligands (BMP, EGFR, JAK/STAT), may be affected. Interestingly, the effect of HSPGs on signaling seems to be dose- and context-dependent. Accordingly, the extracellular accumulation of Wg is only detected when cells express high levels of Dlp. Thus, the activity of Wg is up-regulated in eye disc cells (5), whereas it seems to be down-regulated in wing blade. Moreover, our data show that

Dpp signaling can be activated or repressed in the wing blade cells, suggesting that the outcome of signaling may be different in the same population of cells. Considering the opposite effects of *dally* and *dlp* on Dpp signaling, we suggest that the balance between Dally and Dlp is important to define autonomously the effects on signaling in each developmental context.

Finally, we show that the hyperplastic phenotypes of Hippo mutants can be partially rescued by the lack of HSPGs, indicating the requirement of these proteins for the overgrowth displayed by these mutants. However, we have to emphasize that their role seems to be merely permissive and only required to modulate cell proliferation. Moreover, the up-regulation of HSPGs could have different effects on cell proliferation, depending on the combined outcome of all signaling pathways affected in each developmental context. In this regard, it has been reported that high levels of Wg signaling promote cell proliferation in the wing hinge, whereas they constrain the wing blade growth (22, 23).

In summary, we conclude that the regulation of HSPGs via Hippo signaling is essential to ensure the coordinated activity of different signaling pathways. In this context, Ds and Ft through the Hippo signaling pathway control the process of cell proliferation (7, 8) and patterning formation.

Materials and Methods

All fly strains used are described at www.flybase.bio.indiana.edu unless otherwise indicated (see *SI Materials and Methods*). Histology, immunostainings, statistics, and clone generation have been performed following standard protocols. For details, see *SI Materials and Methods*.

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