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Drosophila Dpp Morphogen Movement Is Independent of Dynamin-Mediated Endocytosis but Regulated by the Glypican Members of Heparan Sulfate Proteoglycans

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Summary

The *Drosophila* transforming growth factor β (TGF-β) homolog Decapentaplegic (Dpp) acts as a morphogen that forms a long-range concentration gradient to direct the anteroposterior patterning of the wing. Both planar transcytosis initiated by Dynamin-mediated endocytosis and extracellular diffusion have been proposed for Dpp movement across cells. In this work, we found that Dpp is mainly extracellular, and its extracellular gradient coincides with its activity gradient. We demonstrate that a blockage of endocytosis by the dynamin mutant *shibire* does not block Dpp movement but rather inhibits Dpp signal transduction, suggesting that endocytosis is not essential for Dpp movement but is involved in Dpp signaling. Furthermore, we show that Dpp fails to move across cells mutant for *dally* and *dally-like* (*dly*), two *Drosophila* glypican members of heparin sulfate proteoglycan (HSPG). Our results support a model in which Dpp moves along the cell surface by restricted extracellular diffusion involving the glypicans Dally and Dly.

Introduction

One important issue in developmental biology is how cells in a developing field acquire their positional information to determine their fates. Secreted signaling molecules of the TGF-β, Wnt, and Hedgehog (Hh) families have been shown to play essential roles in cell fate specification during development. In many developmental contexts, they act as morphogens that emanate from localized sources and form extracellular gradients that differentially regulate cell fates in a concentration-dependent manner (Cadigan, 2002; Gurdon and Bourillot, 2001; Lawrence and Struhl, 1996; Tabata and Takei, 2004; Teleman et al., 2001; Vincent and Dubois, 2002). The problem of how morphogen gradients arise in tissues has attracted intensive studies in recent years. However, the molecular mechanisms of morphogen transport are still unclear. Here, we use *Drosophila* Decapentaplegic (Dpp) morphogen as a model system to address this problem.

dpp encodes a member of the TGF-β superfamily (Padgett et al., 1987; Raftery and Sutherland, 1999) and acts as a long-range morphogen during wing development (Entchev et al., 2000; Lecuit et al., 1996; Muller et al., 2003; Nellen et al., 1996; Tanimoto et al., 2000; Teleman and Cohen, 2000). *Drosophila* wings arise from wing imaginal discs that are subdivided into anterior (A) and posterior (P) compartments. During wing disc development, *dpp* is expressed in a stripe of cells along the anterior-posterior (A-P) compartment boundary (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Posakony et al., 1990; Tabata and Kornberg, 1994). Dpp acts as a long-range morphogen for the A-P axis to control the growth and patterning of both anterior and posterior wing cells. Supporting the role of Dpp as a morphogen, Dpp signaling was shown to induce the expression of target genes such as *spalt* (*sal*) and *optomotor-blind* (*omb*) at distinct threshold concentrations (Lecuit et al., 1996; Nellen et al., 1996). Dpp activity was also shown to repress the transcription of *brinker* (*brk*), a transcription factor that counteracts responses to Dpp (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999). Subsequent studies have directly demonstrated Dpp activity and ligand gradients. First, the Dpp activity gradient can be monitored by visualizing the phosphorylated form of Mothers against *dpp* (pMAD), a cytoplasmic transducer of Dpp signaling (Tanimoto et al., 2000). Second, the Dpp ligand gradient has been visualized directly using GFP-Dpp fusion proteins that retain signaling activity (Entchev et al., 2000; Teleman and Cohen, 2000). Furthermore, recent work demonstrated that the extracellular Dpp morphogen gradient can be converted into nuclear outputs by targeting to silencer elements that generate an inverse transcriptional gradient of *brk* (Muller et al., 2003).

While the function of Dpp as a morphogen during wing development is well established, how Dpp moves across cells to generate its morphogen gradient is not fully understood. Two general models have been considered for Dpp transport (Cadigan, 2002; Gurdon and Bourillot, 2001; Tabata and Takei, 2004; Teleman et al., 2001; Vincent and Dubois, 2002). Dpp movement could occur by extracellular diffusion mechanism(s). Alternatively, Dpp movement could occur by mechanisms of active transport. One mechanism for this latter model proposes that Dpp morphogen moves across cells by a transcytosis mechanism in which receiving cells internalize the Dpp ligand through Dynamin-mediated endocytosis and then recycle it to the cell surface, thus allowing Dpp to spread along tissue (Entchev et al., 2000; Gonzalez-Gaitan, 2003). Using GFP-Dpp, Entchev et al. found that GFP-Dpp forms a long-range concentration gradient, while, in contrast, secreted GFP is unable to generate a stable concentration gradient (Entchev et al., 2000). They showed that much of the GFP-Dpp is in intracellular punctate particles with very little extracellular GFP-Dpp detected. They further demonstrated that, if the spreading of Dpp is challenged with a patch of dynamin mutant cells defective in endocytosis, the Dpp ligand is unable to pass these mutant cells and forms a shadow with reduced Dpp found behind the mutant clone. These observations led to the proposal that endocytosis is required for the long-range movement of Dpp (Entchev

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et al., 2000). However, Lander et al. (2002) proposed on theoretical grounds that diffusive mechanisms of Dpp morphogen transport are much more plausible than non-diffusive ones. They argued that a defect in endocytosis could lead to excess receptor on the cell surface and thus prevent Dpp diffusion by trapping the ligand on these receptors (Lander et al., 2002). Therefore, the mechanism(s) by which Dpp forms a concentration gradient is still unresolved. Does Dpp move across cells through endocytosis or diffusion? Further work is necessary to fully understand Dpp morphogen gradient formation.

One class of cell surface molecules that may regulate Dpp distribution is the heparan sulfate proteoglycans (HSPG) (Bernfield et al., 1999; Lin and Perrimon, 2003; Nybakken and Perrimon, 2002; Perrimon and Bernfield, 2000). HSPGs consist of a protein core to which heparan sulfate (HS) glycosaminoglycan (GAG) chains are attached. Glypicans represent the main cell surface HSPGs, which are linked to the plasma membrane by a glycosyl phosphatidylinositol (GPI) linker. *Drosophila* has two glypican members, Dally and Dally-like (Dly) (Lin and Perrimon, 2003; Nybakken and Perrimon, 2002). In addition, one *syndecan* (Johnson et al., 2004; Spring et al., 1994; Steigemann et al., 2004) and one *perlecan* (Voigt et al., 2002) were identified (Lin and Perrimon, 2000, 2003; Perrimon and Bernfield, 2000). Previous studies have demonstrated roles of Dally and Dly in Wingless (Wg) and Hedgehog (Hh) morphogen distribution (Baeg et al., 2001; Han et al., 2004b). Genetic studies have also implicated the involvement of Dally in Dpp signaling in imaginal disc development (Jackson et al., 1997; Nakato et al., 1995). Interestingly, recent work has demonstrated that *dally* expression in the wing disc is controlled by the same molecular pathways that regulate the expression of the Dpp receptor *thickveins* (*tkv*) (Fujise et al., 2003). Furthermore, elevated Dally expression can enhance Dpp signaling in a cell-autonomous manner. These results led the authors to propose that Dally acts as a Dpp coreceptor in the wing disc (Fujise et al., 2003). However, *dally* hypomorphic alleles used in these experiments are viable, and the defects associated with *dally* are very mild. In particular, the analyses on Dally's role in Dpp morphogen distribution were conducted in *dally* homozygous larvae (Fujise et al., 2003). Since Dally is also involved in Hh signaling (Han et al., 2004b), *dally* homozygous larvae are expected to have reduced Dpp expression. Therefore, their observed results are complicated by reduced Dpp expression in *dally* homozygous larvae (Fujise et al., 2003). Thus, the molecular mechanism(s) of how Dally or/and other HSPGs regulate Dpp distribution remains to be determined.

To fully understand the molecular mechanisms of Dpp morphogen gradient formation during wing development, we have examined the respective roles of Dynamin-mediated endocytosis and HSPGs in Dpp movement and its signaling. In this report, we provide compelling evidence supporting a model in which Dpp movement is not through Dynamin-mediated endocytosis but rather through extracellular diffusion mediated by the HSPGs Dally and Dly. Using GFP-Dpp, we found that Dpp is mainly extracellular, and its extracellular gradient coincides well with its activity gradient. We further demonstrate that a blockage of endocytosis by the dynamin mutant *shibire* (*shi*) does not block Dpp movement but rather inhibits Dpp signaling, suggesting that Dynamin-mediated endocytosis is not required for Dpp movement. Finally, we show that extracellular Dpp molecules fail to move across *dally-dly* double mutant cells. These findings led us to propose that Dpp moves along the cell surface by restricted diffusion involving the HSPGs Dally and Dly.

Results

The Extracellular Dpp Gradient Coincides with its Activity Gradient

To dissect molecular mechanisms of Dpp morphogen movement during wing development, we first sought to examine the extracellular Dpp distribution using GFP-Dpp, which is functional and therefore can be used as a surrogate for Dpp (Entchev et al., 2000; Teleman and Cohen, 2000). A previous study by Teleman and Cohen demonstrated that the majority of mature GFP-Dpp signaling molecules can be digested by a limited amount of proteinase K, suggesting that they are extracellular (Teleman and Cohen, 2000). However, the extracellular GFP-Dpp gradient was not examined in this study. By contrast, Entchev et al. found that only very weak extracellular GFP-Dpp staining was observed when examined using an extracellular staining protocol that stains only extracellular molecules (Entchev et al., 2000) (see Experimental Procedures for details).

To determine whether Dpp morphogen is mainly extracellular or intracellular, we reexamined the extracellular GFP-Dpp distribution using the identical extracellular staining protocol utilized by Entchev et al. (2000) and a different anti-GFP antibody (see Experimental Procedures for details). We reasoned that, if most of the mature Dpp molecules are extracellular as judged by biochemical analysis (Teleman and Cohen, 2000), we would detect broad extracellular Dpp staining. We expressed UAS-GFP-Dpp by dpp^{Gal4} and examined the extracellular GFP-Dpp gradient (Figures 1A and 1B). We also costained the wing disc with a PS1 antibody that recognizes the phosphorylated form of Mad (p-MAD) (Tanimoto et al., 2000) (Figure 1C). Interestingly, a very broad distribution of the extracellular GFP-Dpp was observed (Figure 1B). Importantly, the extracellular GFP-Dpp gradient coincides well with pMAD gradient (Figures 1C and 1D) (Tanimoto et al., 2000). In particular, we observed reduced extracellular GFP-Dpp levels in Dpp-expressing cells where pMAD levels were also reduced due to the inhibitory effect by Hh signaling (Tanimoto et al., 2000). We obtained similar results when another slightly different GFP-Dpp construct was used (data not shown) (Entchev et al., 2000; Teleman and Cohen, 2000). Furthermore, we observed similar extracellular Dpp distribution using the same anti-GFP antibody as in the previous study (Entchev et al., 2000) (see Experimental Procedures and Supplemental Figure S1 at http://www.cell.com/cgi/content/full/119/2/231/DC1/ for details). Together with the previous biochemical studies (Teleman and Cohen, 2000), our results suggest that the

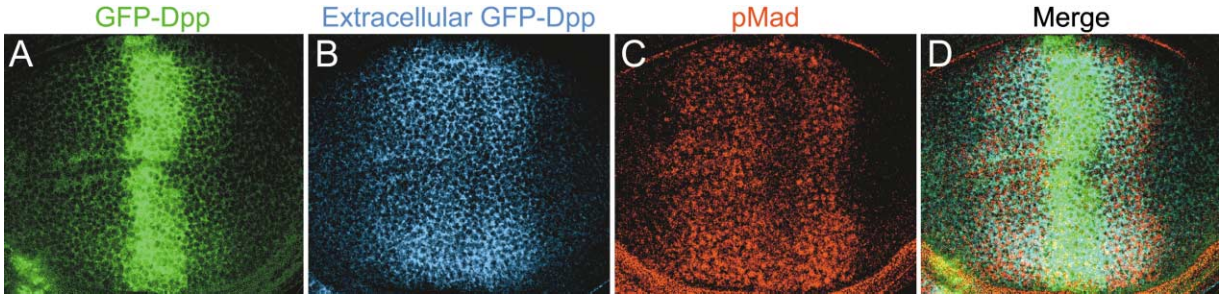


Figure 1. Distribution of the Extracellular GFP-Dpp Gradient in the Wing Disc

All the wing discs presented in this and other figures were derived from third instar larvae. Anterior is toward left, and dorsal is upwards. In a dpp^{d14}/dpp^{d12} mutant wing disc rescued by *GFP-Dpp* under control of dpp^{Gal4} (genotype: dpp^{d14}/dpp^{d12}-*UAS-Dpp-GFP;* $dpp^{Gal4}/+$), the GFP autofluorescence of GFP-Dpp (without staining) forms a gradient (A). Extracellular GFP-Dpp (B) can be visualized by an extracellular staining protocol using a mouse monoclonal anti-GFP antibody. The Dpp activity gradient was visualized by p-MAD staining using the PS1 antibody (C). A merge of the GFP fluorescence of GFP-Dpp, the extracellular GFP-Dpp staining, and the p-MAD staining is shown in (D). The extracellular Dpp staining appears as a gradient, with the higher levels close to the GFP-Dpp expressing cells and the lower levels far from them. Noticeably, the level of extracellular GFP-Dpp on the surface of GFP-Dpp-expressing cells is lower than that of neighboring nonexpressing cells. This pattern correlates well with the Dpp activity gradient represented by pMAD staining (C and D).

extracellular GFP-Dpp gradient reflects the Dpp activity gradient.

Dpp Signaling Is Reduced Cell Autonomously in Dynamin-Defective Cells but Not in Wild-Type Cells Behind Them

The broad distribution of the extracellular GFP-Dpp and its similarity to p-MAD distribution raised a possibility that most of the Dpp molecules may be transported through an extracellular route rather than an intracellular route mediated by endocytosis. To test this, we first examined the activity of Dpp signaling in wing discs bearing clones mutant for $shibire^{ts1}$ (shi^{ts1}). *shi* encodes the *Drosophila* homolog of the dynamin GTPase and is required for endocytosis (Chen et al., 1991; van der Bliek and Meyerowitz, 1991). We reasoned that, if endocytosis is required for Dpp transport, Dpp movement from its source toward distal regions would be blocked by $shibire^{ts1}$ mutant cells. In this case, wild-type cells adjacent and distal to $shibire^{ts1}$ mutant cells would have little or no Dpp and therefore have reduced Dpp signaling. shi^{ts1} mutant clones were generated by the FLP-FRT method (Golic, 1991; Xu and Rubin, 1993), allowed to grow at the permissive temperature (18°C), and then shifted to 34°C for 5 hr to inactivate Shibire as described previously (Entchev et al., 2000). Under these conditions, p-MAD levels were diminished in shi^{ts1} mutant cells in a cell-autonomous fashion (Figure 2). Interestingly, p-MAD levels were not reduced in the wild-type cells behind the shi^{ts1} clones (Figures 2B–2E; shown by turquoise arrows). In fact, in some cases, we even observed slightly enhanced p-MAD activity in the wild-type cells behind the shi^{ts1} clones (Figures 2E; shown by a turquoise arrow). Virtually identical results were obtained when the level of Sal, a target of Dpp signaling, was examined (see Supplemental Figure S2 on the *Cell* web site). These results argue that Dynamin-mediated endocytosis is required for Dpp signaling. Importantly, cells behind shi^{ts1} mutant clones still receive Dpp signaling, suggesting that Dpp is likely able to move across shi^{ts1} mutant cells.

Extracellular Dpp Can Move across Dynamin-Defective Cells

To confirm that Dpp can move across Dynamin-defective cells as suggested from the above experiments, we performed two subsequent experiments. First, we examined extracellular Dpp levels in wing discs bearing shi^{ts1} clones. Dpp-GFP was expressed by Dpp^{Gal4}, and extracellular GFP-Dpp was analyzed. Consistent with the above observations, extracellular GFP-Dpp levels were not reduced on the wild-type cells behind or adjacent to shi^{ts1} mutant clones (Figures 3A′–3D′; shown by white arrows), supporting the view that extracellular GFP-Dpp can move across shi^{ts1} mutant cells to reach the adjacent wild-type cells. Interestingly, extracellular GFP-Dpp even accumulated on the shi^{ts1} mutant cell surface in a cell-autonomous fashion (Figures 3A′–3D′), suggesting that Dynamin-mediated endocytosis is normally required for downregulating extracellular Dpp levels.

In the second experiment, we analyzed extracellular Dpp movement in larvae homozygous for shi^{ts1}. Gal4 activity in *Drosophila* is thermosensitive and is very low at 16°C (Brand and Perrimon, 1993). Both *UAS-GFP-Dpp* and *UAS-dynamin* were expressed under control of dpp^{Gal4} in animals homozygous for shi^{ts1}. These flies were kept at 16°C until the third instar larval stage and then shifted to 32°C for 3 hr to inactivate shi^{ts1}. Under these conditions, all cells except Dpp-expressing cells rescued by *UAS-dynamin* were defective in Dynamin-mediated endocytosis. As expected, little extracellular GFP-Dpp was found at 16°C (Figure 3E′). If Dynamin-mediated endocytosis were required for Dpp movement, extracellular GFP-Dpp molecules should be retained and accumulated on Dpp-expressing cells but not in Dpp-receiving cells, when these flies are shifted to 32°C for 3 hr. However, we observed very strong and broad distribution of extracellular GFP-Dpp beyond the Dpp-expressing cells under these conditions (Figure 3F′), demonstrating that GFP-Dpp molecules are able to move across Dynamin-defective cells to reach more lateral regions of the wing disc. Together, these data argue

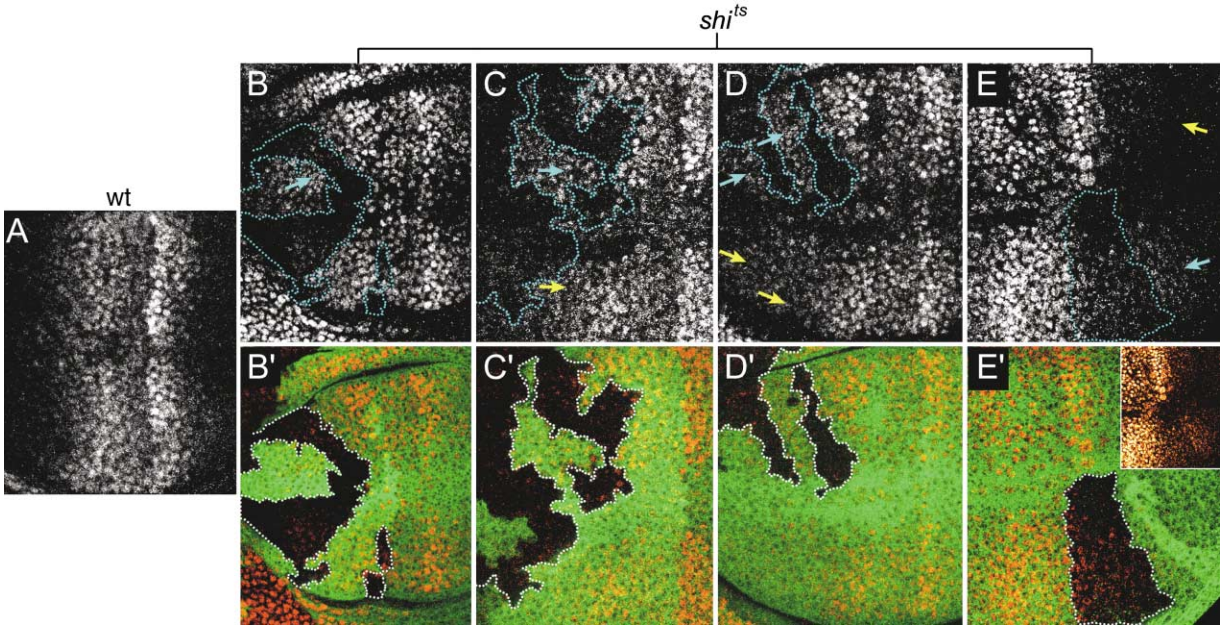


Figure 2. Dpp Signaling Is Diminished Cell Autonomously in *shi^{ts1}* Mutant Cells but Is Not Reduced in the Wild-Type Cells Behind *shi^{ts1}* Mutant Cells

The Dpp activity gradient visualized by p-MAD staining is shown in a wild-type wing disc (A). p-MAD levels are symmetrically distributed in the dorsal and ventral compartments (A). The wing discs carrying *shi^{ts1}* mutant clones were stained by p-MAD and β-gal antibodies. P-MAD staining alone is shown in (B), (C), (D), and (E). Merges of p-MAD staining (red) with β-gal staining (green) were shown in (B′), (C′), (D′), and (E′). *shi^{ts1}* mutant clones are marked by the absence of β-gal staining (green) and labeled with broken lines. p-MAD levels are greatly reduced cell autonomously in *shi^{ts1}* mutant clones (B–E). However, p-MAD levels are not reduced in the wild-type cells behind *shi^{ts1}* mutant clones (shown by turquoise arrows in [C]–[E]) compared with wild-type cells in similar positions in ventral (C and D) or dorsal (E) compartments (shown by yellow arrows in [C]–[E]). p-MAD levels are also maintained in the wild-type cells surrounded by *shi^{ts1}* mutant cells (shown by a turquoise arrow in [B]). We observed slightly elevated p-MAD levels in the wild-type cells behind the *shi^{ts1}* clone shown by a turquoise arrow in (E). In (E′), the inset is a pseudocolored pMad staining shown with higher contrast in order to enhance the visibility of the elevated p-MAD levels in the wild-type cells behind the *shi^{ts1}* clone. We used the same approach to show reduced p-MAD activity and/or extracellular GFP-Dpp levels in wild-type cells behind mutant clones in the remaining figures.

that Dynamin-mediated endocytosis is not required for Dpp movement but is involved in downregulating extracellular Dpp molecules.

Dpp Receptor Tkv Accumulates on Dynamin-Defective Cells

In Dpp-receiving cells, Dpp binds to its receptor Tkv and relays its signal to the receiving cells (Raftery and Sutherland, 1999). The above experiments show that Dpp can move across Dynamin-defective cells, but it fails to transduce its signal to Dynamin-defective cells. One possibility is that Dynamin-mediated endocytosis is required for the internalization of the activated Dpp receptor complex, which might be essential for Dpp signaling. Supporting this view, extracellular GFP-Dpp accumulated on the Dynamin-defective cells (Figure 3). To further characterize this, we examined Tkv levels in the *shi^{ts1}* mutant cells. In the wild-type wing disc, Tkv levels are downregulated by both Hh and Dpp signaling (Lecuit and Cohen, 1998; Tanimoto et al., 2000). *tkv* expression is maintained at low levels in the center of the disc and at higher levels toward the anterior and posterior edges (Figure 4A). Tkv levels were increased cell autonomously on *shi^{ts1}* mutant cells (Figure 4B), demonstrating that Tkv protein is downregulated by Dynamin-mediated endocytosis. To exclude the possibility that the observed Tkv accumulation on *shi^{ts1}* mutant cells is not due to the reduced Dpp signaling activity, we also examined Tkv levels on *shi^{ts1}* mutant cells by shifting larvae from permissive temperature (18°C) to 34°C for only 70 min. Under this experimental condition, Dpp signaling activity assayed by p-MAD and Sal was not reduced in *shi^{ts1}* mutant cells; however, we still observed enhanced Tkv levels on *shi^{ts1}* mutant cells (see Supplemental Figure S3), suggesting that the observed Tkv downregulation by dynamin-mediated endocytosis is independent of Dpp signaling. Collectively, these data suggest that Dynamin-mediated endocytosis is likely to be involved in downregulating extracellular Dpp through the receptor-mediated internalization, thereby shaping the Dpp activity gradient in the wing disc; however, Dynamin-mediated endocytosis is not essential for Dpp movement across cells.

The HSPGs Dally and Dly Have Both Cell-Autonomous and -Nonautonomous Effects on Dpp Signaling in the Wing Disc

HSPGs have been shown to regulate Wg and Hh morphogen distribution (Baeg et al., 2001; Bellaiche et al., 1998; Han et al., 2004b; The et al., 1999); however, their role in Dpp morphogen gradient formation is less understood. We therefore investigated whether HSPGs are

essential for Dpp movement. We first examined Dpp signaling in mosaic clones mutant for *sulfateless* (*sfl*) that encodes a heparan sulfate N-deacetylase/N-sulfotransferase required for HS biosynthesis (Baeg et al., 2001; Lin and Perrimon, 1999). Mutations in *sfl* are expected to impair the function of all HSPGs (Lin and Perrimon, 1999, 2000). p-MAD levels were reduced in *sfl* mutant clones (Figures 5A–5C), suggesting that HSPGs are essential for Dpp signaling in receiving cells. Interestingly, pMAD levels were reduced in the wild-type cells behind the *sfl* mutant clones (Figures 5A and 5B; shown by turquoise arrows). This effect can be seen even when mutant clones are only one to two cells wide (Figure 5B). These results contrast with the observations that pMAD levels are not reduced in wild-type cells behind shi^{ts1} clones (Figure 2), suggesting a possible role(s) for HSPGs in Dpp movement.

Next, we investigated which HSPG core proteins are involved in Dpp movement. Previous studies using *dally* hypomorphic alleles have implicated the involvement of Dally in Dpp signaling (Fujise et al., 2003; Jackson et al., 1997; Nakato et al., 1995). We have recently generated *dally* and *dly* null alleles and demonstrated their partially redundant functions in Hh movement (Han et al., 2004b). p-MAD levels were reduced in *dally* mutant clones but were only slightly decreased in *dly* mutant clones (data not shown). However, p-MAD levels were greatly reduced in *dally-dly* double mutant cells (Figures 5D and 5E), suggesting that Dally and Dly are partially redundant in Dpp signaling in the wing disc. Importantly, we also observed reduced levels of p-MAD in wild-type cells adjacent and distal to *dally-dly* mutant clones (Figures 5D and 5E; shown by turquoise arrows). Both the cell-autonomous and -nonautonomous effects of *dally-dly* clones on Dpp signaling are comparable to those of *sfl*. Collectively, these data suggest that the HSPGs Dally and Dly play a role in Dpp movement and its signaling.

It is interesting to note that, within *sfl* or *dally-dly* mutant clones, p-MAD levels are still maintained in the first row of the mutant cells immediately adjacent to wild-type cells facing Dpp expressing cells, suggesting that *sfl* or *dally-dly* mutant cells are able to receive and response to Dpp signaling but unable to transport Dpp molecules (Figures 5A–5E). Similar results were observed for Hh signaling in cells mutant for HSPGs (Han et al., 2004b).

Extracellular Dpp Fails to Move across *sfl* or *dally-dly* Mutant Cells

To further examine the role of HSPGs in Dpp movement, we analyzed the extracellular Dpp gradient in discs bearing *sfl* or *dally-dly* mutant clones. Extracellular GFP-Dpp levels were significantly reduced in *sfl* (Figures 6A–6C″) or *dally-dly* (Figures 6D–6F″) mutant clones. Importantly, extracellular Dpp levels were also reduced in wild-type cells behind the *sfl* (Figures 6A′, 6B′, and 6C′; shown by white arrows) or *dally-dly* (Figures 6D′, 6E′, and 6F′; shown by white arrows) mutant cells. This effect was not weakened even when mutant clones are only a few cells wide (Figures 6A′, 6B′, and 6F′), demonstrating that Dpp molecules failed to pass across few *sfl* or *dally-dly* mutant cells. Consistent with this, we also observed slightly accumulated extracellular GFP-Dpp in front of *sfl* or *dally-dly* mutant clones (Figures 6C′, 6E′, and 6F′; shown by pink arrows). Together with the cell-nonautonomous effects of *sfl* and *dally-dly* on Dpp signaling, these data argue that, in addition to being required for maintenance of the extracellular Dpp morphogen gradient, the HSPGs Dally and Dly are involved in Dpp movement.

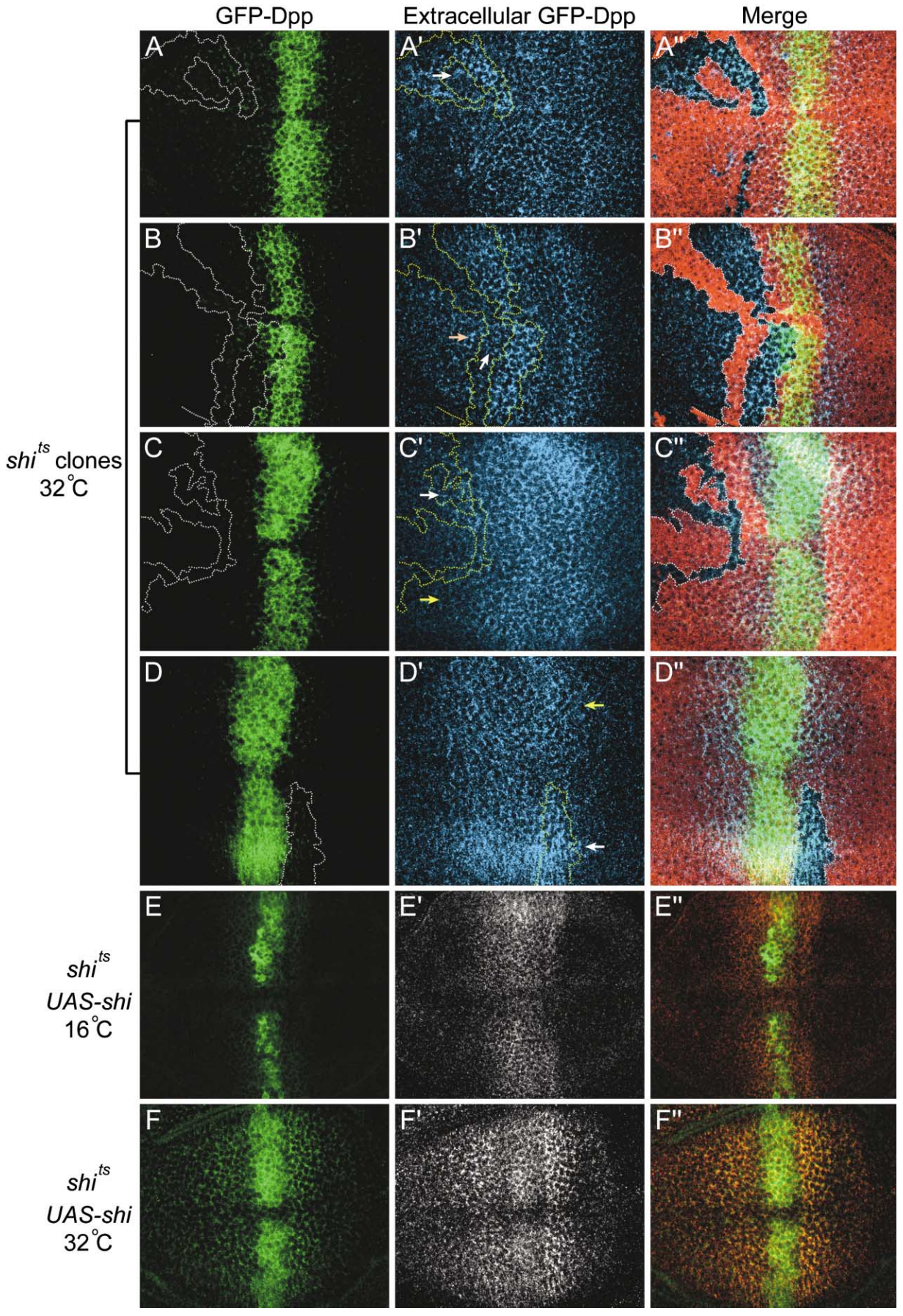
Discussion

Dpp acts as a morphogen that forms a long-range concentration gradient to determine cell fates during wing development. In this work, we found that Dpp is mainly extracellular, and its extracellular gradient coincides with its activity gradient. We further demonstrated that Dynamin-mediated endocytosis is not essential for Dpp movement, although it is required for Dpp signaling. Finally, we provided evidence that the HSPGs Dally and Dly are required for Dpp movement. These observations led us to propose that Dpp moves along the cell surface through restricted diffusion involving the HSPGs Dally and Dly.

The Extracellular Dpp Gradient and Dpp Morphogen Signaling

One new observation in this work is that the extracellular Dpp is broadly distributed in the wing disc. Consistent with our findings, previous biochemical analysis demonstrated that the majority of mature Dpp signaling molecules are extracellular (Teleman and Cohen, 2000). Importantly, the overall shape of the extracellular Dpp gradient coincides well with its activity gradient, suggesting that the extracellular Dpp gradient contributes to Dpp activity gradient in the wing disc. It is important to note that Teleman and Cohen previously showed that GFP-Dpp ligand gradient did not correlate well with p-MAD gradient (Teleman and Cohen, 2000). This discrepancy can be explained by the difference of methods in acquiring data. First, they examined the levels of GFP-Dpp by its intrinsic GFP fluorescence, which will detect both intracellular and extracellular pools of GFP-Dpp. The fluorescence levels of intracellular GFP-Dpp in Dpp-expressing cells are much higher than those of GFP-Dpp in Dpp-receiving cells, which will have very significant impact on the overall distribution chart of GFP-Dpp shown in their paper. In contrast, the extracellular staining protocol allowed us to specifically visualize the distribution pattern of the extracelluar GFP-Dpp without detecting intracellular GFP-Dpp. Second, we have noticed that, in the conventional staining protocol, the extracellular GFP-Dpp can be easily washed away by extensive washing steps. We suspect that, under their condition in which the conventional staining protocol was used, the majority of extracellular GFP-Dpp may have been lost. However, in our extracellular staining protocol, wing discs are incubated with anti-GFP antibody first, and then the extracellular GFP-Dpp bound by anti-GFP antibody is fixed. Thus, we are able to detect majority of extracellular GFP-Dpp. Despite these differences, both studies have found some similar results, for example, the faster drop off of Dpp gradient in the P compartment compared to the A compartment.

The observation of broadly distributed extracellular


GFP-Dpp
Extracellular GFP-Dpp
Merge
A
A'
A''
B
B'
B''
C
C'
C''
D
D'
D''
shi ts clones
32°C
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16°C
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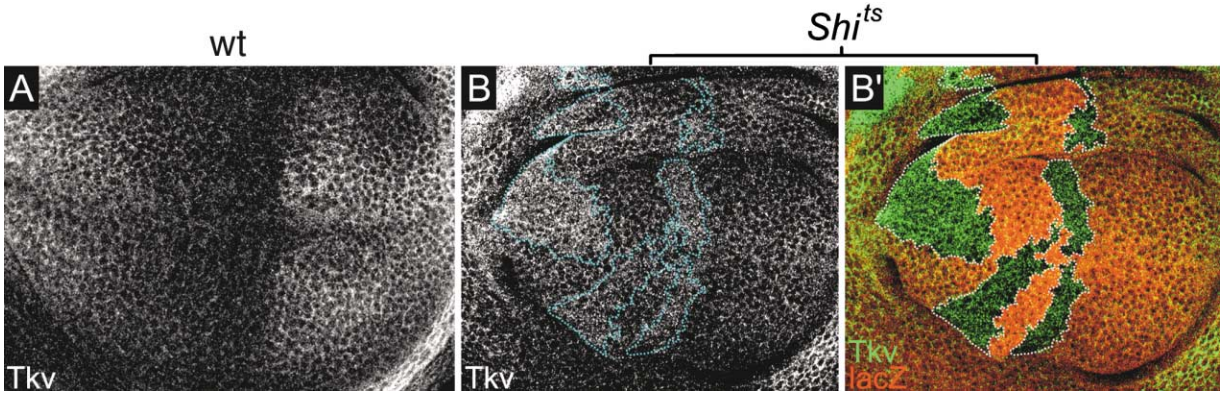


Figure 4. Dpp Receptor Tkv Accumulated in Dynamin-Defective Cells

Dpp receptor Tkv staining is shown in a wild-type wing disc (A) and discs carrying *shi*^{ts1} mutant clones (B and B′). *shi*^{ts1} mutant clones are marked by the absence of β-gal staining (red) and labeled with broken lines. Tkv staining alone is shown in (B), and a merge of Tkv staining with β-gal staining is shown in (B′). In a wild-type wing disc (A), Tkv is maintained at low levels in the center of the disc and at higher levels toward more lateral regions. Tkv levels are increased cell autonomously in *shi*^{ts1} mutant clones (B and B′).

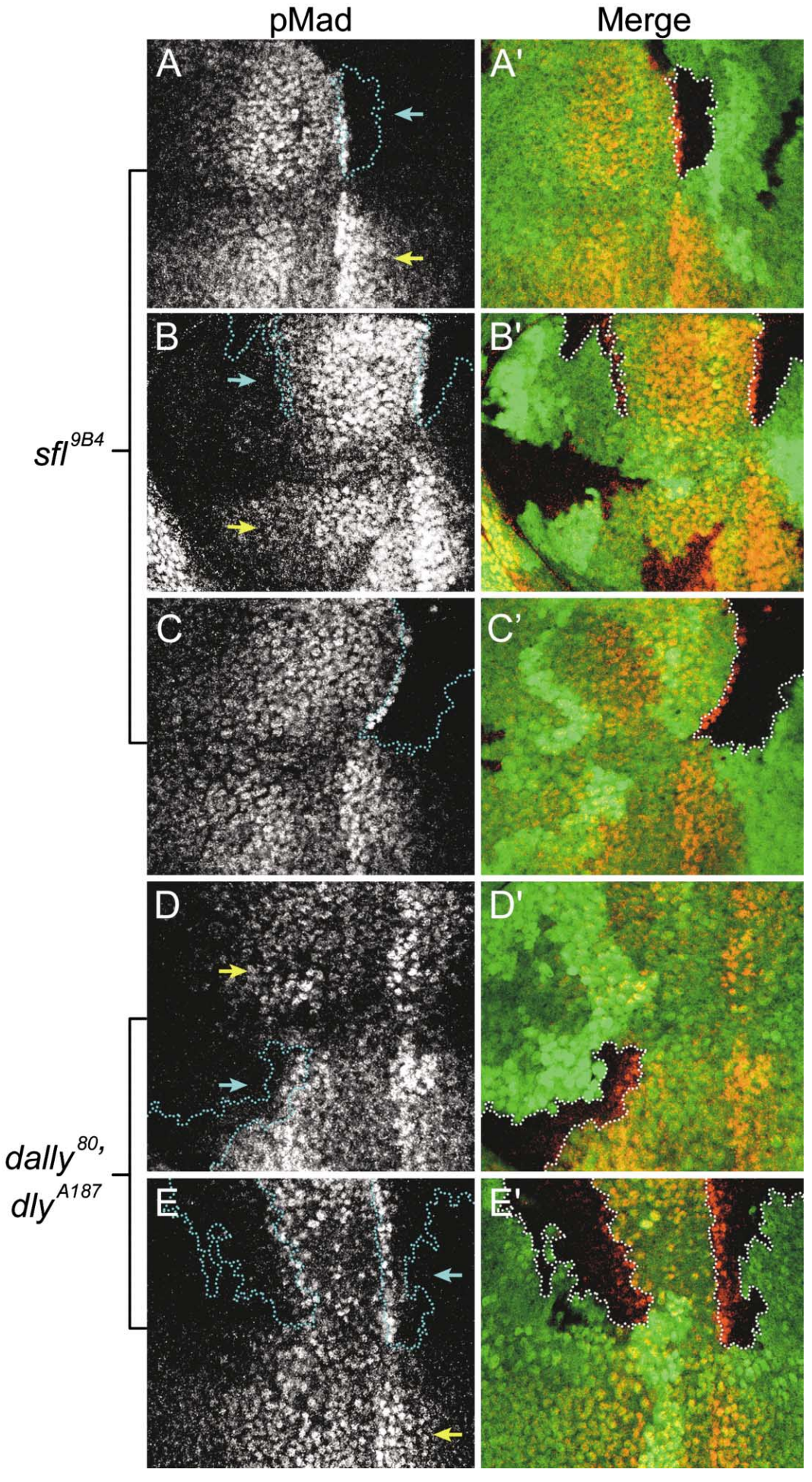
Dpp led us to reexamine the role of Dynamin-mediated endocytosis in Dpp movement and signaling. Our analyses argue that Dynamin-mediated endocytosis is not essential for Dpp movement. First, both Dpp signaling activity and extracellular GFP-Dpp levels are not reduced in the wild-type cells behind the *shi*^{ts1} clones that are defective in endocytosis (Figures 2 and 3). Second, the extracellular GFP-Dpp is also broadly distributed in endocytosis-defective wing discs homozygous for *shi*^{ts1} at nonpermissive temperature (Figure 3). These data demonstrate that Dpp molecules are able to move across Dynamin-defective cells. Finally, we found that extracellular Dpp accumulates on the cell surface of *shi*^{ts1} mutant clones, suggesting that Dpp is able to move into *shi*^{ts1} mutant cells and that Dynamin-mediated endocytosis is normally involved in downregulating levels of the extracellular Dpp. We also do not observe any accumulation of extracellular Dpp on wild-type cells in front of *shi*^{ts1} mutant clones, which would be expected if endocytosis were required for Dpp movement (Cadigan, 2002; Teleman et al., 2001; Vincent and Dubois, 2002).

While Dynamin-mediated endocytosis does not appear to be critical for Dpp movement, we found that Dpp signaling activity is reduced cell autonomously in *shi*^{ts1} mutant cells. This result argues that Dynamin-mediated endocytosis is an essential process for Dpp signaling. Studies in mammalian cell culture system have demonstrated the critical role of Dynamin-mediated internalization of activated TGF-β receptors in TGF-β signaling (Di Guglielmo et al., 2003; Hayes et al., 2002; Panopoulou et al., 2002; Penheiter et al., 2002). SARA (Smad anchor for receptor activation), a FYVE finger protein enriched in early endosomes, was shown to be involved in this process (Hayes et al., 2002; Panopoulou et al., 2002; Penheiter et al., 2002). Although the exact mechanism of endocytosis-mediated TGF-β signaling is still unclear, current data suggest a role of early endosomes as a signaling center for TGF-β (Di Guglielmo et al., 2003; Hayes et al., 2002; Panopoulou et al., 2002; Penheiter et al., 2002). Consistent with this view, Entchev et al. showed that ectopic expression of the dominant-negative form of Rab5 (DRab5S43N) using engrailed-Gal4 led to a reduction of Dpp signaling, while overexpression of Rab5 broadened the Dpp signaling

Figure 3. Extracellular Dpp Can Move across *shi*^{ts1} Mutant Cells in the Wing Disc

UAS-GFP-dpp is expressed under control of *dpp*^{Gal4} in discs carrying *shi*^{ts1} mutant clones (A–D″) or in *shi*^{ts1} homozygous mutant discs (E–F″). The first column (GFP-Dpp) shows the GFP-Dpp autofluorescence signals, which are brighter in the GFP-Dpp-expressing cells than in the Dpp-receiving cells. The second column shows the extracellular GFP-Dpp detected by the extracellular staining protocol using the anti-GFP antibody. The third column shows merges of GFP-Dpp autofluorescence signals with the stained extracellular GFP-Dpp as well as β-gal staining (red in [A″], [B″], [C″] and [D″]). *shi*^{ts1} mutant clones are marked by the absence of β-gal staining and labeled with broken lines. The mutant clones are shown in the anterior (A–A″, B–B″, and C–C″) or in the posterior compartments (D–D″). In all *shi*^{ts1} mutant clones, levels of extracellular GFP-Dpp are increased. The extracellular GFP-Dpp levels are maintained in the wild-type cells surrounded by *shi*^{ts1} mutant cells (shown by a white arrow in [A]) or behind a long strip of *shi*^{ts1} mutant cells (shown by a white arrow in [B]). The extracellular GFP-Dpp levels are also accumulated in a second more anterior *shi*^{ts1} clone (shown by a pink arrow in [B]). The extracellular GFP-Dpp levels are not reduced in the wild-type cells behind *shi*^{ts1} clones (shown by white arrows in [C′] and [D′]) compared with wild-type cells in similar positions in the ventral (C′) or dorsal (D′) compartments (shown by yellow arrows in [C′] and [D′]).

The wing discs from *shi*^{ts1} homozygous mutants expressing both *UAS-GFP-Dpp* and *UAS-Shi* under control of *dpp*^{Gal4} at 16°C (E–E″) or being shifted to 32°C for 3 hr (F–F″). At 16°C, GFP-Dpp expression was very low (E). In this condition, the extracellular GFP-Dpp levels (E′) were very low and were mainly distributed around cells close to *dpp*-expressing cells at the A-P boundary (E′, E″). However, after being shifted to 32°C for 3 hr, GFP-Dpp expression was enhanced (F). The extracellular GFP-Dpp molecules were broadly distributed in both the anterior and posterior compartments (F′). Levels of extracellular GFP-Dpp were enhanced in both the anterior and the posterior compartments. Importantly, the extracellular GFP-Dpp levels were even slightly higher in the anterior and posterior Dpp-receiving cells than those in Dpp-expressing cells. Identical extracellular staining procedure and confocal settings were used to examine the levels of the extracellular GFP-Dpp shown in (E), (E′), and (E″) and in (F), (F′), and (F″).


pMad
Merge
A
A'
B
B'
C
C'
D
D'
E
E'
sfl9B4
dally80,
dlyA187

(Entchev et al., 2000). Rab5 localizes in early endosomes and is required for endosome fusion (Seto et al., 2002). Taken together, we propose that dynamin-mediated endocytosis is not directly involved in Dpp movement but is essential for Dpp signaling. Furthermore, Dynamin-mediated endocytosis can downregulate extracellular Dpp levels, thereby shaping the Dpp morphogen gradient.

Role of the HSPGs Dally and Dly in Dpp Signaling and Movement

To investigate the role of HSPGs in Dpp morphogen gradient formation, we examined Dpp signaling and its extracellular distribution in *sfl* and *dally-dly* mutant clones. Our new findings have clearly demonstrated that *dally* and *dly* are required and partially redundant in Dpp signaling and movement in the wing disc. Two lines of evidence support the role of Dally and Dly in Dpp movement across cells. First, Dpp signaling activity is reduced in cells behind *sfl* or *dally-dly* mutant clones (Figure 5). Second, extracellular Dpp levels are diminished in cells behind *sfl* or *dally-dly* mutant clones (Figure 6). Importantly, we found that *sfl* or *dally-dly* mutant clones of only a few cells wide can effectively block GFP-Dpp movement (Figures 5 and 6), suggesting that Dpp movement does not occur through "free diffusion," by which extracellular Dpp would be expected to move across *sfl* or *dally-dly* mutant cells. Based on these observations, we propose that Dpp moves from cell to cell along the epithelium sheet through restricted diffusion involving Dally and Dly.

If the HSPGs Dally and Dly are indeed involved in Dpp movement, we would expect to observe extracellular GFP-Dpp accumulation in front of *sfl*, *dally-dly* mutant clones (Cadigan, 2002; Tabata and Takei, 2004; Teleman et al., 2001). Indeed, extracellular GFP-Dpp accumulation is visible in front of *sfl* or *dally-dly* mutant clones (Figure 6). Consistent with our observation, Takei et al. (2004) have recently shown that Hh is abnormally accumulated in clones mutant for *tout-velu* (*ttv*) and *brother of tout-velu* (*botv*), two *Drosophila* EXT members involved in HS GAG chain biosynthesis (Bellaiche et al., 1998; Han et al., 2004a; Takei et al., 2004; The et al., 1999). Both Wg and Dpp accumulation in front of *ttv-botv* clones were also observed, albeit less pronounced compared with the case of Hh (Takei et al., 2004). Similarly, in our experiments, extracellular GFP-Dpp accumulation is relatively weak compared with Hh accumulation observed by Takei et al. (2004). One possibility is that extracellular Dpp molecules bound by Dally and Dly in wild-type cells can still be internalized by adjacent *sfl* or *dally-dly* mutant cells through cell-cell contact, leading to a reduction of extracellular Dpp accumulation in front of *sfl* or *dally-dly* mutant cells. Consistent with this view, we noticed that, within *sfl* or *dally-dly* mutant clones, the first row of the mutant cells immediately adjacent to wild-type cells and facing Dpp-expressing cells was still capable of transducing Dpp signaling (Figures 5A–5E).

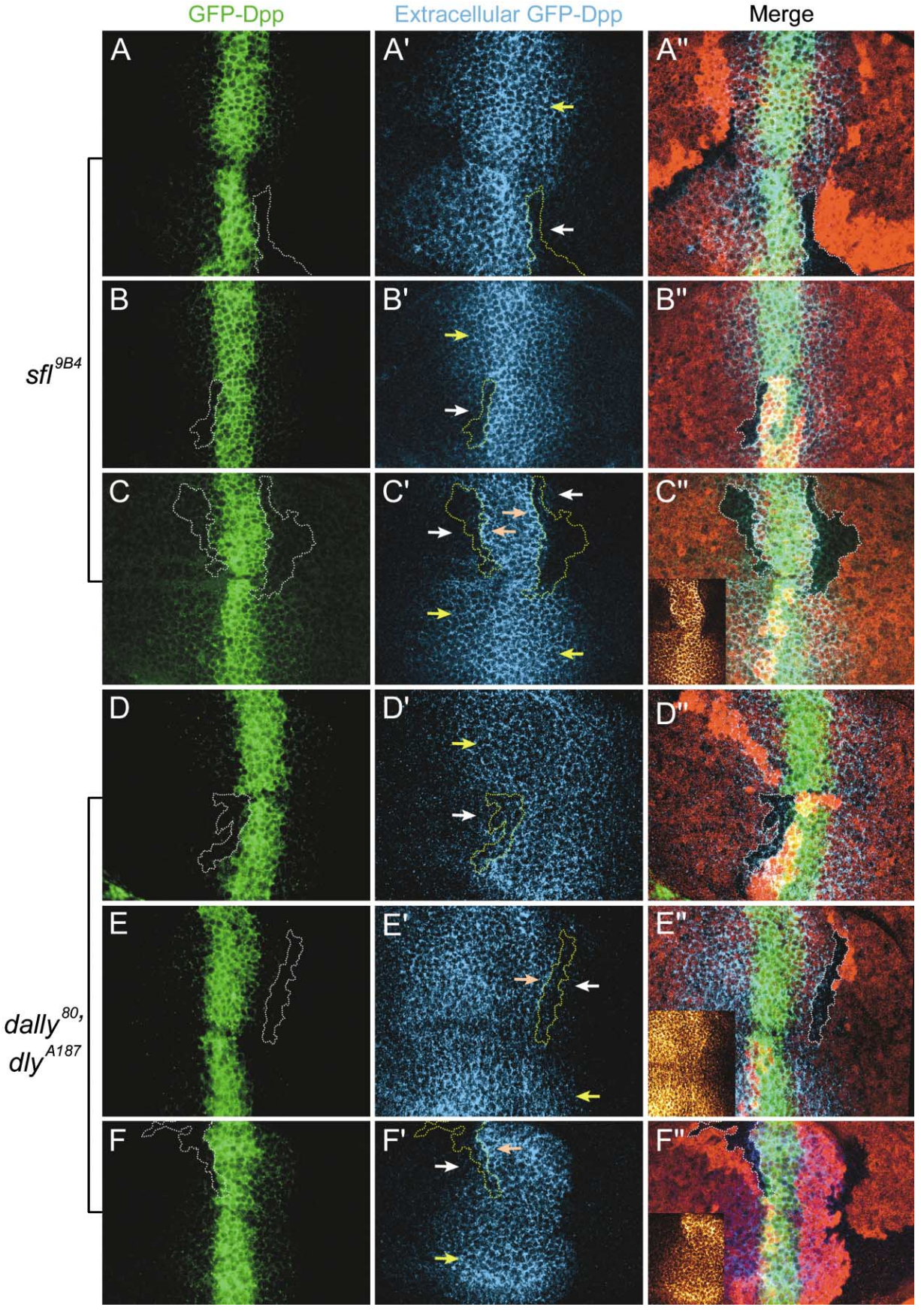
In addition to being required for Dpp movement, Dally and Dly are also essential for Dpp signaling in its receiving cells. We found that Dpp signaling is reduced in *sfl* or *dally-dly* mutant cells. We also observed reduced levels of extracellular Dpp in *sfl* or *dally-dly* mutant clones. Consistent with the results in this work, we and others have shown that clones mutant for *ttv* or *botv* as well as *sister of tout-velu* (*sotv*), members of *Drosophila* EXT, led to reductions in Dpp signaling and its ligand distribution when analyzed by a conventional staining protocol that reveals both extracellular and intracellular Dpp (Bornemann et al., 2004; Han et al., 2004a; Takei et al., 2004). Collectively, these data suggest that the main function of Dally and Dly in Dpp signaling is to maintain and/or concentrate the extracellular Dpp available for Dpp receptors.

A Model of Dpp Morphogen Movement

As discussed above, we demonstrate that Dynamin-mediated endocytosis is not essential for Dpp movement. We further show that Dpp movement is through a cell-to-cell mechanism involving the HSPGs Dally and Dly. On the basis of our findings, we propose the following model (Figure 7). We propose that secreted Dpp molecules in the A-P border are immediately captured by the GAG chains of Dally and Dly on the cell surface located in either the A or P compartments. The differential concentration of Dpp on the cell surface of producing cells and receiving cells drives the displacement of Dpp from one GAG chain to another toward more distant receiving cells. Alternatively, Dpp molecules bound by Dally or Dly could also move along the cell surface through a GPI linkage that is inserted in the outlet leaflet of the plasma membrane and is enriched in raft domains (Simons and Ikonen, 1997). In the receiving cells, Dally and Dly may present Dpp to its receptor, Tkv, that transduces Dpp signal through the Dynamin-mediated internalization process, which further downregulates extracellular Dpp levels and cell surface Tkv. Based on this model, extracellular Dpp and its receptor, Tkv, would be accumulated on the surface of Dynamin-deficient cells (Figure 7A), and extracellular Dpp would be able to move across Dynamin-deficient cells to reach more distal cells. In *sfl* or *dally-dly* mutant clones, extracellular Dpp molecules can not be attached on the cell surface and therefore can not be transferred further to more

Figure 5. The HSPGs Dally and Dly Are Required for Dpp Signaling in the Wing Imaginal Disc

The first column shows p-MAD staining alone. The second column shows merges of p-MAD staining with GFP staining used for visualizing mutant clones. *sfl* (A–C′) or *dally-dly* (D–E′) mutant clones are marked by the absence of GFP staining (green) and labeled with broken lines. pMAD levels are diminished in *sfl* (A–C′) or *dally-dly* (D–E′) mutant clones. Within *sfl* or *dally-dly* mutant clones, p-MAD levels are still maintained in the first row of the mutant cells immediately adjacent to wild-type cells facing Dpp-expressing cells. The reduction of pMAD levels is therefore not apparent in the *sfl* clone located in the anterior compartment of the wing disc (B), since it is only one to two cells wide. Importantly, pMAD levels are reduced in the wild-type cells of more lateral regions behind the *sfl* (A and B) or *dally-dly* (D and E) mutant clones (shown by turquoise arrows) when compared with the wild-type cells in similar positions in the ventral (A, B, and E) or dorsal (D) compartments (shown by yellow arrows in [A], [B], [D], and [E]). This effect can be seen even when mutant clones are only one to two cells wide (B).


GFP-Dpp
Extracellular GFP-Dpp
Merge
A
A'
A''
B
B'
B''
C
C'
C''
D
D'
D''
E
E'
E''
F
F'
F''
sfl9B4
dally80,
dlyA187

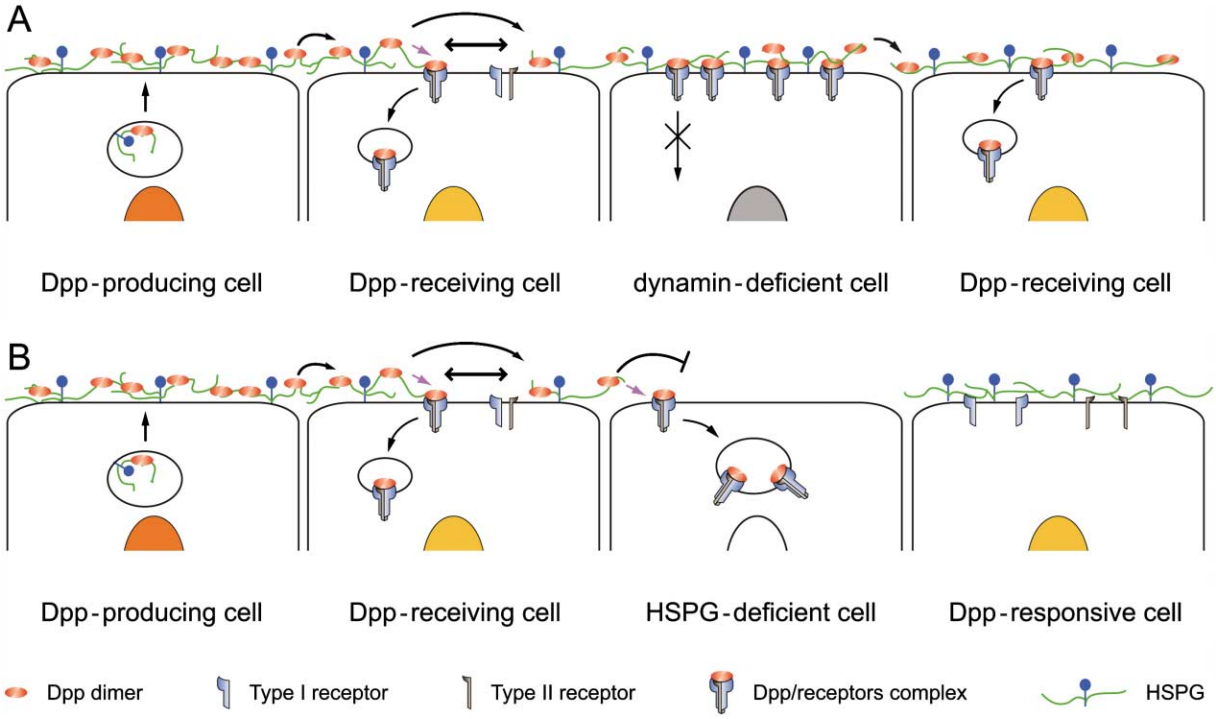


Figure 7. Model of Dpp Movement

A field of cells in wing discs bearing dynamin-deficient cells (A) or HSPG-deficient cells (B) is illustrated. The purple arrow indicates that, upon binding to glypicans, Dpp can be presented to its receptors. The thin black arrows at the top of this diagram indicate the displacement of Dpp from one GAG chain to another GAG chain. The double-headed arrow indicates the lateral movement of glypicans on the cell membrane. Secreted extracellular Dpp molecules can move across Dynamin-deficient cells (A) but fail to move across the HSPG-deficient cells (B) (see text for details).

distal cells (Figure 7B). In this model, endocytosis is not directly involved in Dpp movement; however, through receptor-mediated internalization, Dynamin-mediated endocytosis can downregulate extracellular Dpp levels, thereby shaping the Dpp morphogen gradient. It remains to be determined how Dpp is transferred from one cell to another by the GAG chains of Dally and Dly and whether Dally and Dly play a role in preventing extracellular Dpp from degradation. Further studies are needed to determine whether other mechanisms are also involved in Dpp movement.

Our Data in the Context of Previously Reported Results

Although dynamin-mediated endocytosis was previously proposed to control Dpp movement in the wing disc (Entchev et al., 2000), Lander et al. proposed on the basis of mathematical analysis that diffusive mechanisms of Dpp transport are more plausible than nondiffusive mechanisms (Lander et al., 2002). Our work presented here supports a diffusive mechanism involving HSPGs. We feel that the main differences in the conclusions of our work and the conclusions reached in Entchev et al. (2000) are due to differences in interpretations of the experimental data. First, Entchev et al. also did not observe a reduction in Dpp levels and its signaling activity in the wild-type cells behind *shi*^{ts1} mutant clones (Entchev et al., 2000). This was interpreted as a result of Dpp movement through lateral and downstream neighbor cells. However, we think that this is very unlikely to be the case, since p-MAD staining and extracellular GFP-Dpp levels in the wild-type cells are not re-

Figure 6. Extracellular GFP-Dpp Cannot Bypass HSPGs-Deficient Cells

UAS-GFP-dpp is expressed under control of *dpp*^{Gal4} in discs carrying *sfl* (A–C″) or in *dally-dly* (D–F″) mutant clones. Mutant clones are marked by the absence of DsRed (Red) and labeled with broken lines. The first column (GFP-Dpp) shows the GFP-Dpp autofluorescence signals that are brighter in the GFP-Dpp-expressing cells than in the Dpp-receiving cells. The second column (extracellular GFP-Dpp) shows the stained extracellular GFP-Dpp detected by the extracellular staining protocol using the anti-GFP antibody. The third column shows merges of GFP-Dpp autofluorescence signals with the stained extracellular GFP-Dpp as well as DsRed staining (shown in red). Extracellular GFP-Dpp levels are reduced within the *sfl* (A′, B′, and C′) or *dally-dly* (D′, E′, and F′) mutant clones, although the reduction is weaker in the first one to two rows of cells that are adjacent to the clone boundary and facing the GFP-Dpp-expressing cells. Levels of the extracellular Dpp in regions behind *sfl* ([A′], [B′], and [C′], shown by white arrows) or *dally-dly* ([C′], [D′], and [E′], shown by white arrows) are reduced when compared with wild-type cells in similar positions in the dorsal (A′, B′, and D′) or the ventral (C′, E′, and F′) compartments (shown by yellow arrows). This effect can be seen even when mutant clones are only one to two cells wide (A′, B′, and F′). Extracellular GFP-Dpp accumulated slightly in wild-type cells facing Dpp expressing cells in front of *sfl* or *dally-dly* mutant clones ([C′], [E′], and [F′], shown by pink arrows). In (C″), (E″), and (F″), insets are pseudocolored higher-contrast extracellular GFP-Dpp staining in order to enhance the visibility of the accumulated extracellular GFP-Dpp.

duced even when the wild-type cells are surrounded by *shi*^{ts1} mutant cells (Figures 2 and 3). In this case, Dpp movement from lateral and downstream neighbor cells would be blocked by *shi*^{ts1} mutant cells if Dynamin-mediated endocytosis were required for Dpp movement. By contrast, we show that both Dpp signaling and extracellular GFP-Dpp levels are reduced in wild-type cells behind a strip of cells mutant for *sfl* or *dally-dly* (Figures 5 and 6). Second, Entchev et al. showed that ectopic expression of dominant-negative form of Rab5 (DRab5S43N) using engrailed-Gal4 led to a reduction of the range of Dpp signaling while overexpression of Rab5 broadened the Dpp signaling range (Entchev et al., 2000). This was interpreted as evidence for a role of endocytosis in Dpp movement. However, we show that endocytosis is required for Dpp signaling. In this regard, their results can be simply explained as changes in Dpp signaling in the P compartment rather than alterations in Dpp movement, since the entire posterior wing was targeted (Entchev et al., 2000). Finally, Entchev et al. showed that the amount of GFP-Dpp vesicles in the wild-type cells behind *shi*^{ts1} clones was reduced when the spreading of Dpp was challenged with a patch of dynamin mutant cells (Entchev et al., 2000). In this experiment, larvae bearing *shi*^{ts1} clones were raised at 16°C to minimize the expression of GFP-Dpp and then shifted to 29°C to initiate a wave of GFP-Dpp propagation. However, Lander et al., based on theoretical grounds, demonstrated that elevated levels of receptors can cause the GFP-Dpp shadow in this dynamic condition (Lander et al., 2002). Consistent with this view, we found that Dpp receptor Tkv levels are increased cell autonomously in *shi*^{ts1} clones (Figure 4 and Supplemental Figure S3). Therefore, the GFP-Dpp shadow observed in the dynamic condition is most likely due to elevated Tkv levels in *shi*^{ts1} clones. It is also important to note that, even in this dynamic condition, Dpp signaling activity examined by Sal staining is not reduced in cells behind *shi*^{ts1} clones (Entchev et al., 2000). By contrast, Dpp signaling activity is strikingly reduced in cells behind *sfl* or *dally-dly* mutant clones (Figure 5). In fact, in some cases, we even observed slightly enhanced Dpp signaling in wild-type cells behind the *shi*^{ts1} clones (Figure 2), which may result from accumulated extracellular Dpp at the cell surface of *shi*^{ts1} clones (Figure 3).

Experimental Procedures

Drosophila Stocks

UAS-GFP-Dpp and *dpp*^{Gal4} stocks were described in Entchev et al. (2000) and Teleman and Cohen (2000). *dpp*^{d12}, *dpp*^{d14}, and *shi*^{ts1} are described in FlyBase. *sfl*^{9B4} was described in Baeg et al. (2001) and Lin and Perrimon (1999). *dally*^{80}, *dly*^{A187}, and *hs-DsRed* were described in Han et al. (2004a, 2004b).

Generation of Marked Clones

Clones of mutant cells were generated by the FLP-FRT method (Golic, 1991; Xu and Rubin, 1993) and induced by subjecting first or second instar larvae to a heat shock at 37°C for 2 hr as described (Belenkaya et al., 2002). To induce the expression of DsRed marker, third instar larvae were subsequently subjected to a second heat shock for 90 min at 37°C and allowed to recover for 5 hr at room temperature before immunostaining. To generate *shibire* mutant clones, larvae were allowed to grow at 18°C and were shifted to 34°C for 5 hr prior to fixation and antibody staining. Below, we list the genotypes used in our analyses:

(1) Expression of GFP-Dpp by *dpp*^{Gal4} in the wing disc of *dpp* mutant larvae (Figure 1): *dpp*^{d14}/*dpp*^{d12}*-UAS-GFP-Dpp; dpp*^{Gal4}/+.

(2) *shi*^{ts1} clones marked by absence of LacZ (Figures 2 and 4): *armadillo-LacZ FRT*^{18A}/*shi*^{ts1} *FRT*$^{18A;}$ *hsp70-flp*/+.

(3) *shi*^{ts1} clones marked by absence of LacZ in discs expressing GFP-Dpp by *dpp*^{Gal4} (Figure 3): *armadillo-LacZ FRT*^{18A}/*shi*^{ts1} *FRT*^{18A}*; hsp70-flp/UAS-GFP-Dpp; dpp*^{Gal4}/+.

(4) *sfl*^{9B4} or *dally*^{80}*-dly*^{A187} clones marked by the absence of GFP (Figure 5): *y w hsp70-flp*/+ *or Y; ubiquitin-GFP FRT*^{2A}/*sfl*^{9B4} (or *dally*^{80}*-dly*^{A187}) *FRT*^{2A}.

(5) *sfl*^{9B4} or *dally*^{80}*-dly*^{A187} clones marked by the absence of DsRed (Figure 6): *y w hsp70-flp*/+ or *Y; UAS-GFP-Dpp*/+*; hs-DsRed FRT*^{2A}*/ sfl*^{9B4} (or *dally*^{80}*-dly* ^{A187}) *dpp*^{Gal4} *FRT*^{2A}.

Imaginal Disc Immunostaining

All imaginal discs shown in this report were dissected from mid to late third instar larvae, fixed, and stained with appropriate primary antibodies to mark clones and monitor target gene expression or extracellular Dpp distribution after the induction of clonal markers as described (Han et al., 2004a, 2004b). Primary antibodies were used at the following dilutions: rabbit anti-pMAD (PS1) at 1:10,000 (a gift from T. Tabata) (Persson et al., 1998; Tanimoto et al., 2000), rat anti-Tkv at 1:250 and rat anti-Sal at 1:500 (gifts from S. Cohen) (Teleman and Cohen, 2000), chicken anti-GFP (Chemicon) at 1:2000, and rabbit anti-DsRed at 1:4000 (Clontech). Primary antibodies were detected by fluorescent-conjugated secondary antibodies from Jackson ImmunoResearch Laboratories.

Extracellular Staining Protocol

The extracellular staining protocol is derived from a previous study (Strigini and Cohen, 2000). Third instar larvae were dissected in ice-cold Schneider's M3 medium (Sigma) and incubated with mouse monoclonal anti-GFP antibody for 1 hr on ice. After rinsing three times for a total of 5 min with ice-cold PBS, larvae were fixed in PBS containing 4% formaldehyde at room temperature for 20 min. After fixation, larvae were rinsed four times quickly, washed for 20 min with PBST (PBS plus 0.1% Triton X-100), and preblocked for another 20 min in PBST with 5% normal horse serum. Larvae were incubated with fluorescent-conjugated secondary antibody for 1.5 hr, rinsed four times quickly, and then washed three times (20 min each wash) and mounted. We used mouse monoclonal anti-GFP at 1:200 (MAB3580 from Chemicon). Mounting medium was 70% glycerol containing 2.5% DABCO.

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