Drosophila glypicans Dally and Dally-like shape the extracellular Wingless morphogen gradient in the wing disc

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Summary

***Drosophila* Wingless (Wg) is the founding member of the Wnt family of secreted proteins. During the wing development, Wg acts as a morphogen whose concentration gradient provides positional cues for wing patterning. The molecular mechanism(s) of Wg gradient formation is not fully understood. Here, we systematically analyzed the roles of glypicans Dally and Dally-like protein (Dlp), the Wg receptors Frizzled (Fz) and Fz2, and the Wg co-receptor Arrow (Arr) in Wg gradient formation in the wing disc. We demonstrate that both Dally and Dlp are essential and have different roles in Wg gradient formation. The specificities of Dally and Dlp in Wg gradient formation are at least partially achieved by their distinct expression patterns. To our surprise, although Fz2 was suggested to play an essential role in Wg gradient formation by ectopic expression studies, removal of Fz2 activity does not alter the extracellular Wg gradient. Interestingly, removal of both Fz and Fz2, or Arr causes enhanced extracellular Wg levels, which is mainly resulted from upregulated Dlp levels. We further show that Notum, a negative regulator of Wg signaling, downregulates Wg signaling mainly by modifying Dally. Last, we demonstrate that Wg movement is impeded by cells mutant for both *dally* and *dlp*. Together, these new findings suggest that the Wg morphogen gradient in the wing disc is mainly controlled by combined actions of Dally and Dlp. We propose that Wg establishes its concentration gradient by a restricted diffusion mechanism involving Dally and Dlp in the wing disc.**

Key words: Wingless (Wg), morphogen, Wnt, Heparan sulfate proteoglycans, Dally, Dally-like (Dlp), Frizzled (Fz) receptor, signaling

Introduction

During the development of metazoa, tissue patterning is controlled by a small group of secreted signaling molecules called morphogens. Morphogens are produced by a subset of cells in a tissue and form concentration gradients that provide positional information for cell fate specifications (Gurdon and Bourillot, 2001; Lawrence and Struhl, 1996). Well established morphogens include members of the Wingless (Wg)/Wnt, Hedgehog (Hh) and bone morphogenetic protein (BMP) families. The functions of these morphogens have been well characterized in the *Drosophila* wing disc (Cadigan, 2002; Tabata and Takei, 2004; Teleman et al., 2001; Vincent and Dubois, 2002). However, the mechanisms of their gradient formation are still not fully understood. Here, we focus our studies on the mechanism(s) of Wg morphogen gradient formation in the wing disc.

Wg is the founding member of the Wnt family of secreted proteins and plays essential roles in many developmental processes (Nusse, 2003; Wodarz and Nusse, 1998). In the third instar wing disc, Wg is expressed in two to three cell widths straddling the dorsoventral (DV) compartment boundary. Wg forms a concentration gradient to activate the expression of its target genes including *achaete-scute* (*ac*), *distalless* (*dll*) and *vestigial* (*vg*) in a concentration-dependent manner (Neumann and Cohen, 1997; Strigini and Cohen, 2000; Zecca et al., 1996). Studies from *Drosophila* embryonic epidermis and the wing disc lead to several models to explain Wg movement across a field of cells (Cadigan, 2002; Tabata and Takei, 2004; Teleman et al., 2001; Vincent and Dubois, 2002). Two prevalent models are: (1) diffusion through the extracellular space (Strigini and Cohen, 2000); and (2) planar transcytosis via dynamin-mediated endocytosis (Bejsovec and Wieschaus, 1995; Moline et al., 1999). The diffusion model proposes that Wg movement is through an extracellular route, while planar transcytosis suggests that Wg moves via intracellular routes through dynamin-mediated endocytosis. In addition, Wg movement by membrane fragments called argosomes (Greco et al., 2001) or by retention on the cell surface (Pfeiffer et al., 2000; Pfeiffer et al., 2002) are also proposed. In the wing disc, much of the evidence is consistent with the diffusion model (Strigini and Cohen, 2000). However, currently the exact mechanism(s) of Wg diffusion and the molecules regulating this process are unclear.

Wg relays its signal through two functionally redundant receptors, Frizzled (Fz) and Fz2, both of which belong to the family of seven-pass transmembrane proteins (Nusse, 2003; Wodarz and Nusse, 1998). Arrow (Arr), a member of the LDL

receptor-related protein (LRP) family of proteins, is also required for Wg signaling and has been postulated to act as a co-receptor for Wg (He et al., 2004; Tolwinski et al., 2003; Wehrli et al., 2000). Previously, Cadigan et al. have proposed that Fz2 played essential roles in shaping the Wg morphogen gradient in the wing disc (Cadigan et al., 1998). This conclusion is mainly based on the following observations. First, overexpression of Fz2 in the wing disc stabilizes Wg and expands the range of Wg-target gene expression. Second, Fz2 expression in the wing disc is inhibited by Wg signaling and appears to be an inverse gradient to that of Wg. This inverse gradient of Fz2 is thought to facilitate the spread of Wg into more distant areas. However, subsequent loss-of-function studies demonstrated the functional redundancy of Fz2 and Fz for Wg signaling in both embryos and in the wing disc (Bhanot et al., 1999; Chen and Struhl, 1999). Therefore, the proposed role of Fz2 in Wg gradient formation needs to be further examined by loss-of-function studies. Furthermore, whether Fz and Arr are required for Wg gradient formation is currently unclear.

In recent years, heparan sulfate proteoglycans (HSPGs) have been shown to play roles in the distributions of morphogen molecules (Esko and Selleck, 2002; Lin, 2004). HSPGs are the cell-surface and extracellular matrix molecules composed of a protein core to which heparan sulfate (HS) glycosaminoglycan (GAG) chains are attached (Bernfield et al., 1999; Esko and Selleck, 2002). Glypicans represent the main cell-surface HSPGs that are linked to the plasma membrane by a glycosyl phosphatidylinositol (GPI) linker. *Drosophila* has two glypican members, Division abnormally delayed (Dally) and Dally-like protein (Dlp) (Baeg et al., 2001; Khare and Baumgartner, 2000; Nakato et al., 1995). Our recent studies have demonstrated that both Dally and Dlp are required for Hh and Dpp movement through a restricted diffusion mechanism (Belenkaya et al., 2004; Han et al., 2004b). Regarding Wg morphogen formation, several studies have shown that mutations in genes involved in HS biosynthesis lead to defects in Wg distribution in the wing disc (Baeg et al., 2001; Bornemann et al., 2004; Han et al., 2004a; Luders et al., 2003; Takei et al., 2004). We have previously shown that Dally plays a role in Wg signaling in the wing disc (Lin and Perrimon, 1999). Furthermore, overexpression of Dlp leads to enhanced extracellular Wg levels in the wing disc (Baeg et al., 2001). Together, these studies implicate possible roles of Dally and Dlp in Wg gradient formation; however, their functions and mechanisms in Wg gradient formation have not been examined by loss-of-function study.

Additional evidence for the involvement of HSPGs in Wg distribution comes from studies of *notum* (also called *wingful*) (Gerlitz and Basler, 2002; Giraldez et al., 2002). *notum* encodes a secreted protein that belongs to the α/β-hydrolase superfamily with similarity to pectin acetylesterases. In both embryos and wing discs, *notum* expression mirrors *wg* expression. Mutations in *notum* lead to enhanced Wg levels and increased Wg signaling, while overexpression of *notum* blocks Wg signaling activity (Gerlitz and Basler, 2002; Giraldez et al., 2002). In *Drosophila* S2 cells, co-expression of Dally and Dlp with Notum reduces the amount of Dally and altered the electrophoretic mobility of Dlp, respectively. These data suggest that Notum may regulate Wg signaling by modulating Dlp or/and Dally (Giraldez et al., 2002). However, it remains to be determined whether Dally or Dlp or other HSPGs are indeed the substrate(s) for Notum in vivo.

In this study, we attempt to systematically analyze the relative roles of the glypicans (Dally and Dlp), the Wg receptors (Fz and Fz2) and the Wg co-receptor Arr in Wg gradient formation in the wing disc. We show that Dally and Dlp are essential and have different roles in setting up the Wg gradient. The specificities of Dally and Dlp in Wg gradient formation are at least partially achieved by their distinct expression patterns. To our surprise, extracellular Wg levels are not reduced, but rather enhanced, in *fz-fz2* or *arr* mutant cells. We further demonstrate that Wg protein fails to move across a stripe of *dally-dlp* mutant cells. Together, these new findings suggest that the Wg morphogen gradient in the wing disc is mainly controlled by Dally and Dlp. We propose that Wg establishes its concentration gradient by a restricted diffusion mechanism involving Dally and Dlp.

Materials and methods

Fly strains

The following null or amorphic alleles of *dally*, *dlp*, *dsh*, *notum*, *fz*, *fz2*, *arr*, *sfl* and *botv* were used: $dally^{80}$ and dlp^{A187} (Han et al., 2004b); dsh^{75} (Manoukian et al., 1995); wif^{141}(Gerlitz and Basler, 2002); fz^{H51}, fz^{P21}(Jones et al., 1996); $fz2^{C1}$ (Chen and Struhl, 1999); arr^{2} (Wehrli et al., 2000); sfl^{9B4} (Lin and Perrimon, 1999); and $botv^{103}$ (Han et al., 2004a) (see http://flybase.bio.indiana.edu). $dally^{80}$ and dlp^{A187} are null alleles for *dally* and *dlp*, respectively (Han et al., 2004b). dlp^{A203} harbors a nonsense mutation at amino acid 484 and was used to generate dlp^{A187}/dlp^{A203} adult flies. $dally^{P2}$ is a *dally-lacZ* line used to visualize *dally* expression (Nakato et al., 1995). The following UAS constructs were used to overexpress the corresponding transgenes in the posterior compartment of the wing disc: *UAS-dlp* (Baeg et al., 2001), *UAS-dally* (Tsuda et al., 1999), *UAS-fz* and *UAS-fz2* (Zhang and Carthew, 1998), and *UAS-gfp-arr*. UAS-gfp-arr contains the Wg signal peptide (amino acids 1-37) followed by GFP protein and six Myc tags fused with Arr in which the signal peptide (amino acids 1-54) of Arr is deleted. It can fully rescue the *arr*-null embryos derived from *arr* germline. The following lines were also used: en^{Gal4}, hh^{Gal4} and $tub1\alpha\text{-}Gal80^{ts}$ (McGuire et al., 2003; McGuire et al., 2004). *Act>y+>Gal4 UAS-GFP* (Ito et al., 1997) was used to drive the expression of $UAS\text{-}arm^{act}$ (Pai et al., 1997) and $UAS\text{-}TCF^{DN}$ (van de Wetering et al., 1997) in random clones marked with GFP.

Antibodies and immunofluorescence

Fixation of imaginal discs and antibody staining procedures were performed as described (Belenkaya et al., 2002). Extracellular Wg staining was performed as described (Baeg et al., 2001; Strigini and Cohen, 2000). Primary antibodies were used at the following dilutions: mouse anti-Wg 4D4 at 1:3 (Iowa Developmental Studies Hybridoma Bank; IDSHB), rat anti-Ci (1:10) (Motzny and Holmgren, 1995), rabbit anti-β-gal at 1:500 (Cappel), mouse anti-β-gal at 1:3000 (Roche Molecular Biochemicals), mouse anti-Dlp at 1:50 (Lum et al., 2003), rabbit anti-Wg at 1:500 (Reichsman et al., 1996), mouse anti-Hnt at 1:50 (IDSHB), guinea pig anti-Hrs (full-length) at 1:200 (Lloyd et al., 2002) and rabbit anti-GFP Alexa Fluor 488 at 1:1000 (Molecular Probes). The primary antibodies were detected by fluorescent-conjugated secondary antibodies from Jackson ImmunoResearch Laboratories.

Generation of marked clones and ectopic expression

Clones of mutant cells in the wing disc 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 1 hour. Ectopic expression of Dlp and Fz2-GPI in the P compartment

of the wing disc were induced by heat-shock at 30°C for 24 hours prior to dissection (see genotypes below). Mutations in *Minute* on chromosomes 2R and 3L were used to generate large clones of cells mutant for certain genes. The use of *Minute*$^{-/+}$ does not perturb normal Wg distribution (see Fig. S1 in the supplementary material). Below, we list the genotypes used in our analyses.

Clones mutant for *dally*, *dlp*, *dally-dlp* and *sfl* marked by the absence of GFP (Figs 1, 8)

y w hsp70-flp/+ or Y; hsp70-Myc-GFP M(3)i^{55} *FRT*^{2A}*/dally*^{80} *FRT*^{2A}
y w hsp70-flp/+ or Y; hsp70-Myc-GFP M(3)i^{55} *FRT*^{2A}*/dlp*^{A187} *FRT*^{2A}
y w hsp70-flp/+ or Y; hsp70-Myc-GFP M(3)i^{55} *FRT*^{2A}*/dally*^{80}*-dlp*^{A187} *FRT*^{2A}
y w hsp70-flp/+ or Y; hsp70-Myc-GFP M(3)i^{55} *FRT*^{2A}*/sfl*^{9B4} *FRT*^{2A}

Clones expressing Arm^{act} and TCF^{DN} marked by GFP (Fig. 2)

y w hsp70-flp/UAS-arm$^{act;}$ *act>y*$^{+}$*>Gal4 UAS-GFP/+*
y w hsp70-flp/+ or Y; act>y^{+}*>Gal4 UAS-GFP/UAS-TCF*^{DN}

Clones mutant for *notum*, *dally-notum*, *dlp-notum* marked by the absence of GFP (Figs 2, 3)

y w hsp70-flp/+ or Y; hsp70-Myc-GFP M(3)i^{55} *FRT*^{2A}*/wif*^{141} *FRT*^{2A}
y w hsp70-flp/+ or Y; hsp70-Myc-GFP M(3)i^{55} *FRT*^{2A}*/dally*^{80} *wif*^{141} *FRT*^{2A}
y w hsp70-flp/+ or Y; hsp70-Myc-GFP M(3)i^{55} *FRT*^{2A}*/dlp*^{A187} *wif*^{141}*FRT*^{2A}

Clones mutant for *dally-notum* marked by yellow on the wing (Fig. 3H)

y w hsp70-flp/Y; y^{+} *FRT*^{2A}*/dally*^{80} *wif*^{141} *FRT*^{2A}

Clones mutant for *dsh*, *fz*, *fz2*, *fz1-fz2*, *sfl-fz1-fz2*, *arr*, and *arr-botv* marked by the absence of GFP (Figs 2, 4, 5)

y w hsp70-flp ubiquitin-GFP FRT^{101} */dsh*^{75} *FRT*^{101}
y w hsp70-flp/+ or Y; hsp70-Myc-GFP M(3)i^{55} *FRT*^{2A}*/fz*^{H51} *FRT*^{2A}
y w hsp70-flp/+ or Y; hsp70-Myc-GFP M(3)i^{55} *FRT*^{2A}*/fz2*^{C1} *FRT*^{2A}
y w hsp70-flp/+ or Y; hsp70-Myc-GFP M(3)i^{55} *FRT*^{2A}*/fz*^{H51} *fz2*^{C1} *FRT*^{2A}
y w hsp70-flp/+ or Y; hsp70-Myc-GFP M(3)i^{55} *FRT*^{2A}*/fz*^{P21} *fz2*^{C1} *FRT*^{2A}
y w hsp70-flp/+ or Y; hsp70-Myc-GFP M(3)i^{55} *FRT*^{2A}*/sfl*^{9B4} *fz*^{H51} *fz2*^{C1} *FRT*^{2A}
y w hsp70-flp/+ or Y; FRT^{G13} *ubiquitin-GFP M(2)58F/FRT*^{G13} *arr*^{2}
y w hsp70-flp/+ or Y; FRT^{G13} *ubiquitin-GFP M(2)58F/FRT*^{G13} *arr*^{2} *botv*^{103}

Discs overexpressing Dlp in the P compartment (Fig. 6)

tub1α-Gal80^{ts}*/UAS-dlp; hh*^{Gal4}*/+*

Image processing

For the analyses of overexpressing Wg receptors (Fz and Fz2), Wg co-receptor (Arr) and HSPGs (Dally and Dlp) in the P compartment of the wing disc, the raw data of extracellular Wg staining were exported in tiff format. The plot values were measured and calculated from selected regions in Scion Image. The plot values were then used to generate plot profiles in Microsoft Excel. For each experiment, plot profiles were generated from at least three discs and similar results were obtained. For Fig. 8, the profiles of Wg distribution were generated similarly.

Results

Dally and Dlp have both distinct and overlapping roles in shaping the extracellular Wg gradient

To examine the roles of Dally and Dlp in Wg gradient formation, we analyze the extracellular Wg distribution in wing discs bearing clones mutant for *dally* and *dlp*. Loss of *dally* or *dlp* activity in the wing disc leads to perturbation of the Wg gradient (Fig. 1A-E′). However, the Wg gradient defects associated with *dally* and *dlp* mutant clones are noticeably different. Extracellular Wg is reduced in *dally* clones (Fig. 1A-C′). This defect is rather cell non-autonomous. First, in the same DV compartment, extracellular Wg levels do not have sharp shifts at the boundary between wild-type cells and *dally* mutant cells. Instead, it forms a wedge-shaped gradient from wild-type cells to *dally* mutant cells, and a reduced extracellular Wg level can even be seen in wild-type cells that are close to clone boundaries (indicated by yellow arrows in Fig. 1B,C). Second, the reduction of extracellular Wg in small *dally* clones is less obvious (data not shown). Extracellular Wg levels are also reduced in *dlp* clones (Fig. 1D-E′). However, this effect is only obvious when the mutant cells are four to five cell diameters away from the DV boundary. The extracellular Wg levels are essentially unchanged in this seven- to 10-cell zone surrounding the DV boundary (Fig. 1D-E′).

Extracellular Wg is not completely lost in either *dally* or *dlp* mutant clones. We suspected that this may be due to the partially redundant functions of Dally and Dlp, which was observed for Hh and Dpp signaling (Belenkaya et al., 2004; Han et al., 2004b). To test this, we examined the extracellular Wg levels in clones mutant for both *dally* and *dlp* (referred to as *dally-dlp* hereafter). Indeed, we observed a stronger reduction of the extracellular Wg in the *dally-dlp* clones when compared with *dally* or *dlp* clones (Fig. 1F-G′). This effect is independent of the positions of *dally-dlp* clones. Collectively, these data suggest that Dally and Dlp have both distinct and overlapping roles in shaping the Wg gradient.

Dlp forms an inverse gradient to the Wg gradient and its expression is down-regulated by Wg signaling

The distinct extracellular Wg defects associated with *dally* and *dlp* clones suggest that Dally and Dlp may play different roles in Wg gradient formation. One possibility is that Dally and Dlp may be differentially expressed in the wing disc, and their levels may determine their relative contributions to Wg gradient formation. We tested this possibility by examining the expression patterns of Dally and Dlp in the wing disc. Previously, Fujise et al. demonstrated that *dally* mRNA distribution was virtually identical to that observed with a *dally-lacZ* line (Fujise et al., 2003). *dally* expression in the wing pouch is induced by Hh and Wg signaling, but repressed by Dpp signaling (Fujise et al., 2001; Fujise et al., 2003). As shown in Fig. 2A-A′, *dally* expression visualized by β-galactosidase (β-gal) staining in the *dally-lacZ* line is higher in two regions in the wing pouch. The first is a zone at the DV boundary, the width of which increases with distance from the anteroposterior (AP) compartment boundary. The second is a stripe of cells at the AP boundary, which corresponds to Hh signaling cells (determined by Ci accumulation) (Fig. 2A′).

We then examined Dlp expression using the anti-Dlp antibody (Lum et al., 2003). In the wing pouch, Dlp protein is distributed in most cells except in a zone centered at the DV boundary (Fig. 2B,B′). This zone is about 7-10 cell diameters in width, corresponding well to the region where removal of *dlp* does not alter extracellular Wg levels (Fig. 1D-E′). As Dlp expression appears to be a gradient inverse to the Wg gradient

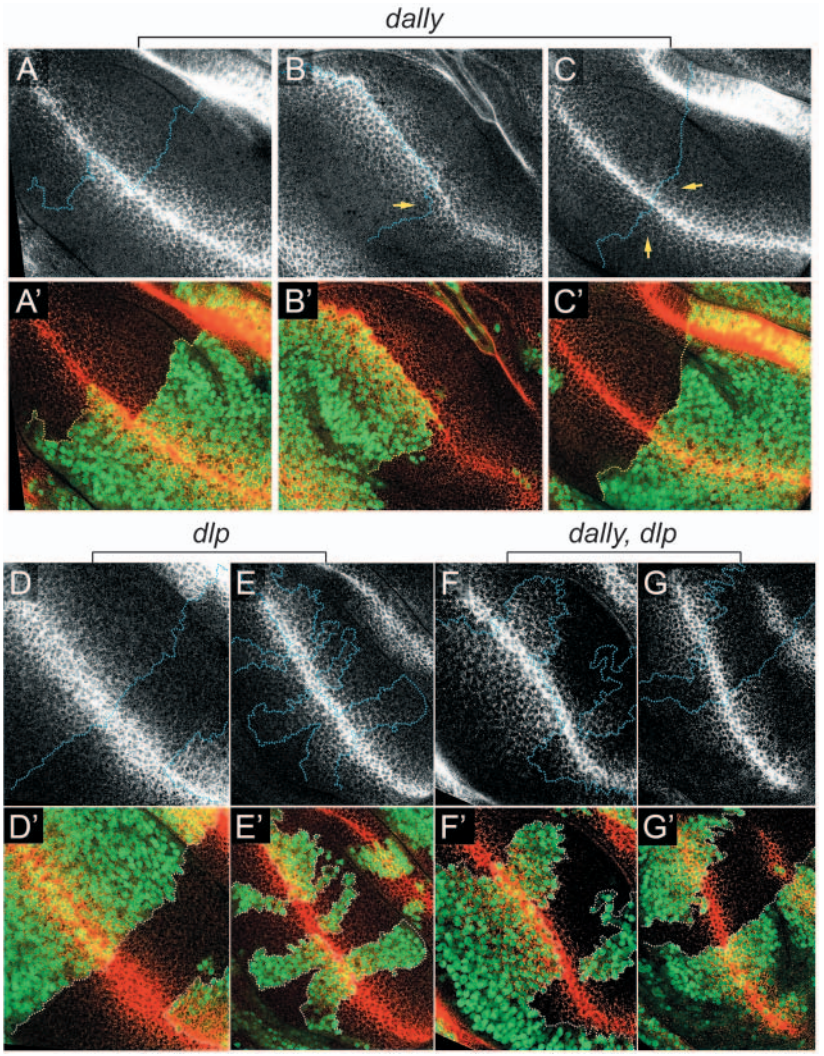

Fig. 1. Dally and Dlp are required for the proper distribution of extracellular Wg. All wing discs are oriented dorsal top-right, anterior top-left. All mutant clones are marked by the absence of GFP and outlined by broken lines. (A-C′) Extracellular Wg staining in discs bearing large clones mutant for *dally*. Extracellular Wg is significantly reduced in the clones in a cell non-autonomous manner. The yellow arrows indicate the wild-type cells that have reduced extracellular Wg level. (D-E′) Extracellular Wg staining in discs bearing large clones mutant for *dlp*. Within the clones, extracellular Wg is reduced outside of a zone that is 7-10 cell diameters wide and centered at the DV boundary. (F-G′) Extracellular Wg staining in discs bearing large clones mutant for both *dally* and *dlp*. Extracellular Wg level is greatly reduced in these clones, and the reduction is stronger than that of *dally* or *dlp* alone.

along the DV axis, we suspect that Dlp expression may be negatively regulated by Wg signaling. Indeed, two lines of evidence support this view. When Wg signaling is activated in random clones by overexpressing a constitutively active form of Armadillo (Arm^{act}) (Pai et al., 1997), Dlp levels are autonomously reduced within the clones (Fig. 2C,C′). Conversely, when Wg signaling is inhibited in random clones by overexpressing a dominant-negative form of TCF (TCF^{DN}) (van de Wetering et al., 1997) or in clones mutant for *disheveled* (*dsh*) (Nusse, 2003; Wodarz and Nusse, 1998). Dlp is induced autonomously within the clones (Fig. 2D-E′). Collectively, our data argue that Dlp expression is negatively regulated by Wg signaling. Taken together with the observation that Dally is upregulated by Wg signaling at DV boundary (Fujise et al., 2001), our new results suggest that the relative contributions of Dally and Dlp in Wg gradient formation are controlled, at least in part, by their expression levels.

Notum modulates Wg signaling mainly by downregulating Dally activity in the wing disc

In the wing disc, Notum is expressed at the DV boundary and its expression is induced by high levels of Wg signaling (Gerlitz and Basler, 2002; Giraldez et al., 2002). Wg signaling activity is enhanced in the *notum* mutant wing disc, suggesting that Notum acts as an inhibitor for Wg (Gerlitz and Basler, 2002; Giraldez et al., 2002). Although biochemical experiments demonstrated the ability of Notum in modifying both Dally and Dlp in cultured S2 cells (Giraldez et al., 2002), it is unclear whether Notum acts on Dally or Dlp in vivo to regulate Wg signaling in the wing disc. As Dlp is absent or expressed at very low levels at the DV boundary where Notum is expected to have the highest activity, we suspect that high levels of Notum at DV boundary may downregulate Dlp protein levels, thereby inhibiting Wg signaling. To test this possibility, we examined Dlp protein levels in *notum* clones. The Dlp protein level is not enhanced in a big clone mutant for *notum* (Fig. 2E,E′) and is also not elevated in the notum homozygous wing disc (data not shown), demonstrating that downregulation of Dlp protein levels at DV boundary is not due to Notum activity.

As Dally is highly expressed at the DV boundary, we anticipated that Dally may be a more relevant target for Notum in the wing disc. The following lines of evidence support this view. First, we generated a double mutant for *dally* and *notum* (*dally-notum*). If Dally is downstream to, and the major substrate for, Notum, *dally-notum* mutant phenotypes should resemble those of the *dally* mutant alone. Loss of *notum* in the wing disc leads to upregulation of Wg signaling and therefore induction of ectopic sense organ precursor (SOP) cells, which can be visualized by Hindsight (Hnt) staining (Giraldez et al., 2002) (Fig. 3B), whereas loss of *dally* leads to reduced numbers of SOP cells (Fig. 3C). In *dally-notum* mutant clones, we never saw ectopic Hnt staining. Instead, we consistently observed reduced numbers of SOP cells in large clones (Fig. 3D). Second, both *notum* and *dally* homozygous animals can develop into adult flies at a low frequency. As a result of elevated Wg signaling, the *notum* wing bears many ectopic thick mechanosensory bristles and curved chemosensory bristles at the wing margin (Fig. 3F), whereas the *dally* wings show a reduced numbers of chemosensory bristles (Fig. 3G). In the *dally-notum* clones, we could not find extra bristles but rather observed a loss of chemosensory bristles (Fig. 3H). Together, the *dally-notum* mutant exhibits similar phenotypes

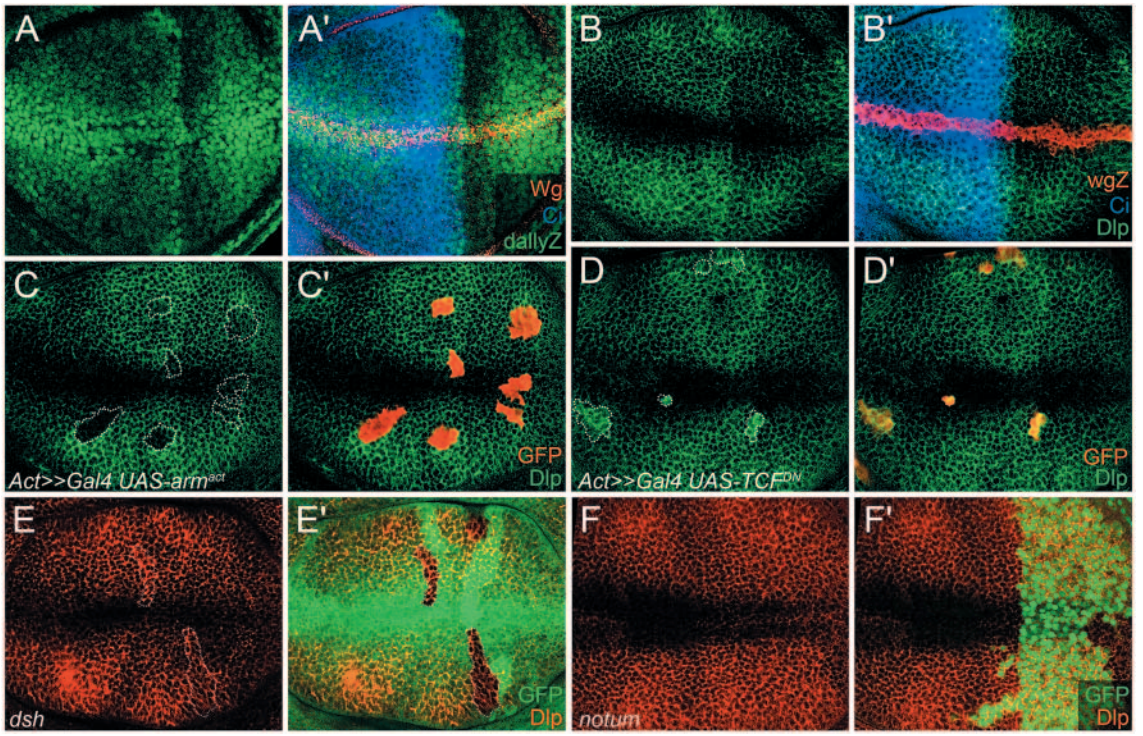

Fig. 2. The expression of Dally and Dlp in the wing disc. All discs are oriented dorsal upwards, anterior leftwards. (A-B′) Dally and Dlp expression in third instar wing imaginal discs visualized by *dally-lacZ* expression (green in A and A′) and Dlp staining (green in B and B′), respectively. The AP and DV compartment boundaries are visualized by Ci staining (blue in A′ and B′) and Wg or *wg-lacZ* expression (red in A′ and B′), respectively. (C-D′) Dlp staining (green) in discs carrying random clones expressing either the active form of Arm (Arm^{act}) (C,C′) or the dominant-negative form of TCF (TCF^{DN}) (D,D′). The clones are marked by GFP (red) and outlined with broken lines. The expression of Dlp is reduced in clones expressing Arm^{act}, and increased in clones expressing TCF^{DN}. (E-F′) Dlp staining (red) in discs carrying *dsh* clones (E,E′) or a large *notum* clone covering the entire A compartment (F,F′). Both *dsh* and *notum* mutant cells are marked by the absence of GFP (green) (E′,F′). Dlp levels are increased in *dsh* clones, but not altered in the absence of *notum* function.

to those seen in the *dally* mutant, suggesting that Notum regulates Wg signaling mainly by modifying Dally activity in the wing disc.

We further asked what role Dlp may play in Wg signaling. Previous work has shown that ectopic expression of Dlp in the wing disc inhibits Wg signaling, suggesting that Dlp may negatively regulate Wg signaling in the wing disc (Baeg et al., 2001). Our loss-of-function analysis suggests that this is indeed the case. Ectopic SOP cells can occasionally be seen in *dlp* mutant clones (Fig. 3I). Consistent with this, escapers of *dlp* homozygous flies ($dlp^{A187/A203}$) show extra mechanosensory bristles (Fig. 3K). However, *dlp* is a weaker inhibitor of Wg signaling than *notum,* as removal of *dlp* results in fewer ectopic SOP cells or extra bristles than removal of *notum*. Importantly, we found that removal of both *notum* and *dlp* phenocopies *notum* loss of function (Fig. 3J,L). Together with the absence/low level expression of Dlp at DV boundary, we argue that Notum acts mainly on Dally to downregulate Wg signaling in the wing disc. Our data also suggest that Wg downregulates Dlp expression to ensure its high levels of signaling activity at the DV boundary.

Extracellular Wg levels are not reduced, but enhanced in the absence of Wg receptors (Fz and Fz2) or the co-receptor Arr

Next, we examined the roles of Wg receptors Fz and Fz2, as well as co-receptor Arr in Wg morphogen gradient formation. The extracellular Wg gradient is not altered in *fz* mutant clones in the late-third instar larvae wing discs (Fig. 4A,A′). However, we occasionally observed slightly reduced extracellular Wg levels in *fz* mutant clones in mid-third instar larval discs (data not shown). This reduction must be transient because it is of low frequency (13%, n=30) and disappears in late-third instar discs. However, we never observed reduced extracellular Wg levels in *fz2* mutant clones (Fig. 4B,B′), suggesting that Fz2 is not essential for extracellular Wg gradient distribution. Our loss-of-function results do not support the previous view for a role of Fz2 in Wg distribution, which was mainly based on ectopic expression data (Cadigan et al., 1998). One possibility is that Fz and Fz2 may be functionally redundant in extracellular Wg distribution. To test this, we examined extracellular Wg levels in clones mutant for both *fz* and *fz2* (*fz-fz2*). To our surprise, extracellular Wg levels are not reduced, but enhanced within the mutant clones (Fig. 4C,C′). Wg refines its own expression domain at the DV boundary and loss of Wg signaling in cells adjacent to the Wg-expressing cells leads to ectopic Wg expression (Rulifson et al., 1996). This is likely to be the case for the enhanced extracellular Wg in *fz-fz2* cells adjacent to the DV boundary (yellow arrows in Fig. 4C). However, the increased extracellular Wg far away from the DV boundary (indicated by turquoise arrows in Fig. 4C) in *fz-fz2* mutant cells is unlikely to be due to ectopic Wg expression as

these cells are more than six cells away from the Wg stripe at the DV boundary. We also examined the role of the Wg co-receptor Arr in Wg gradient formation. Similar to *fz-fz2* clones, *arr* clones result in accumulated extracellular Wg (Fig. 4D,D′).

The increased extracellular Wg levels in *fz-fz2* or *arr* clones may be caused by at least two possibilities. First, as Dlp is repressed by Wg signaling, *fz-fz2* or *arr* clones may upregulate the levels of Dlp, thereby indirectly enhancing the extracellular Wg levels. Alternatively, removal of Wg receptor Fz and Fz2 or co-receptor *arr* may impair the ability of cells to internalize Wg. The following lines of evidence support the first possibility. First, as expected, Dlp is upregulated in *fz-fz2* or *arr* clones (Fig. 5A,A′,C,C′). Second, we examined extracellular Wg levels in clones mutant for *fz-fz2* and deficient for HSPGs. As the *dlp* and *fz* loci are too close to each other, we were unable to generate a *dlp-fz-fz2* triple mutant chromosome. Instead, we used *sulfateless* (*sfl*) which encodes a heparan sulfate N-deacetylase/N-sulfotransferase required for HS biosynthesis (Baeg et al., 2001). Mutations in *sfl* are expected to impair most, if not all, functions of HSPGs, including Dlp (Baeg et al., 2001; Lin and Perrimon, 1999). Indeed, extracellular Wg is reduced in the *sfl-fz-fz2* clones (Fig. 5B,B′). Similarly, extracellular Wg is reduced in clones mutant for both *arr* and *botv*, which encodes a heparan sulfate co-polymerase required for HSPG biosynthesis (Fig. 5D,D′) (Han et al., 2004a; Takei et al., 2004). These results suggest that enhanced extracellular Wg levels in *fz-fz2* or *arr* clones are HSPG dependant. Finally, we also examined extracellular Wg levels in clones mutant for *dsh*. Dsh is an intracellular protein acting downstream of the Wg receptor, therefore it will probably not interfere with Wg internalization. *dsh* mutant clones disrupt Wg signaling, thereby causing Dlp upregulation (Fig. 2E,E′). Importantly, we observed very striking accumulation of extracellular Wg in clones mutant for *dsh* (Fig. 5E-F′). Extracellular Wg accumulation can be clearly seen in *dsh* clones which are located far away from DV boundary (Fig. 5E-F′). Collectively, our results provide compelling evidence that accumulated extracellular Wg protein in *Fz-fz2* or *arr* mutant clones are mainly resulted from upregulated Dlp, which probably further stabilizes extracellular Wg protein on the cell surface.

We also examined internalized Wg vesicles in *fz-fz2* or *arr* clones by co-staining Wg and the endosome marker Hrs (Lloyd et al., 2002). We observed significant amount of internalized Wg vesicles in *fz-fz2* and *arr* clones, suggesting that Wg internalization still occur in the absence of Fz and Fz2 activities or Arr activity (data not shown).

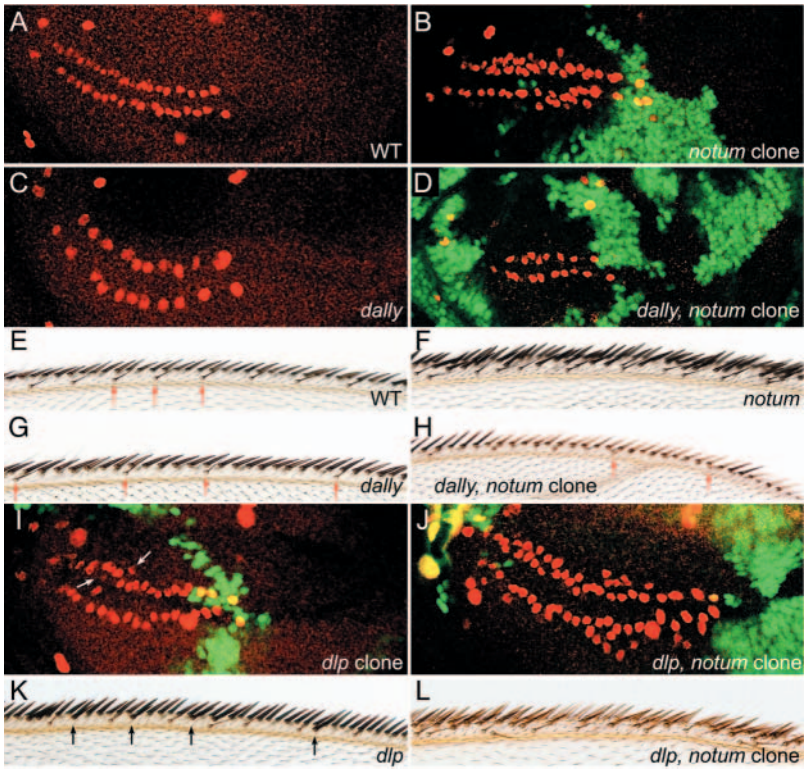

Fig. 3. Notum suppresses Wg signaling mainly through Dally. (A-D) Hnt staining (red) in a wild-type disc (A), a homozygous *dally* disc (C) and discs carrying clones mutant for *notum* (B) or *dally-notum* (D). Wg-dependent Hnt expression marks sense organ precursor (SOP) cells, which will give rise to bristles on the future wing margin. In the wild-type disc, SOP cells are in two rows abutting Wg-expressing cells. Each row contains 20-22 cells. In the clone mutant for *notum*, Hnt is ectopically expressed such that the rows are doubled, while in the *dally* disc, the number of SOP cells in each row is reduced to 9-11. In the clone mutant for *dally-notum*, the number of SOP cells is also reduced. (E-H) Anterior wing margins of wild-type adult (E), *notum* escaper (F), *dally* escaper (G) and a wing carrying large clones of *dally-notum* double mutant (marked by yellow colour) (H). The dorsal view of the wild-type wing margin reveals two rows of sensory bristles: densely packed stout mechanosensory bristles and interspersed chemosensory bristles. Three chemosensory bristles are indicated by red arrows to show the spacing. The wing of the *notum* escaper has many ectopic bristles of both types, while that of *dally* has a reduced number of chemosensory bristles, as visualized by the increased spacing (red arrows). The *dally-notum* clone does not have ectopic bristles but has increased spacing between chemosensory bristles (red arrows). (I,J) Hnt staining in discs carrying large clones mutant for either *dlp* (I) or *dlp-notum* (J). Occasionally, few ectopic SOP cells can be seen in *dlp* clones (white arrows), while the *dlp-notum* clone has many ectopic SOP cells. (K,L) Anterior wing margins of $dlp^{A187/A203}$ escaper (K) and a wing carrying a large clone of *dlp-notum* double mutant (L). The *dlp* wing has a few ectopic stout mechanosensory bristles (black arrows), while many ectopic bristles of both types were produced in the *dlp-notum* clone.

The capacities of HSPGs (Dally and Dlp), the Wg receptors (Fz and Fz2) and the co-receptor Arr in modulating the extracellular Wg gradient

Previous studies and our loss-of-function data suggest that different HSPGs and Wg receptors may have differential intrinsic abilities in influencing Wg distribution. To compare their differences, we ectopically expressed individual HSPGs and Wg receptors in the P compartment of the wing disc by using en^{Gal4} or hh^{Gal4} drivers, and then compared the extracellular Wg distribution in the A and P compartments. The signal intensity of extracellular Wg staining was averaged and plotted from two comparable regions selected from the A and P compartments. In the wild-type disc, the patterns of the extracellular Wg gradient are

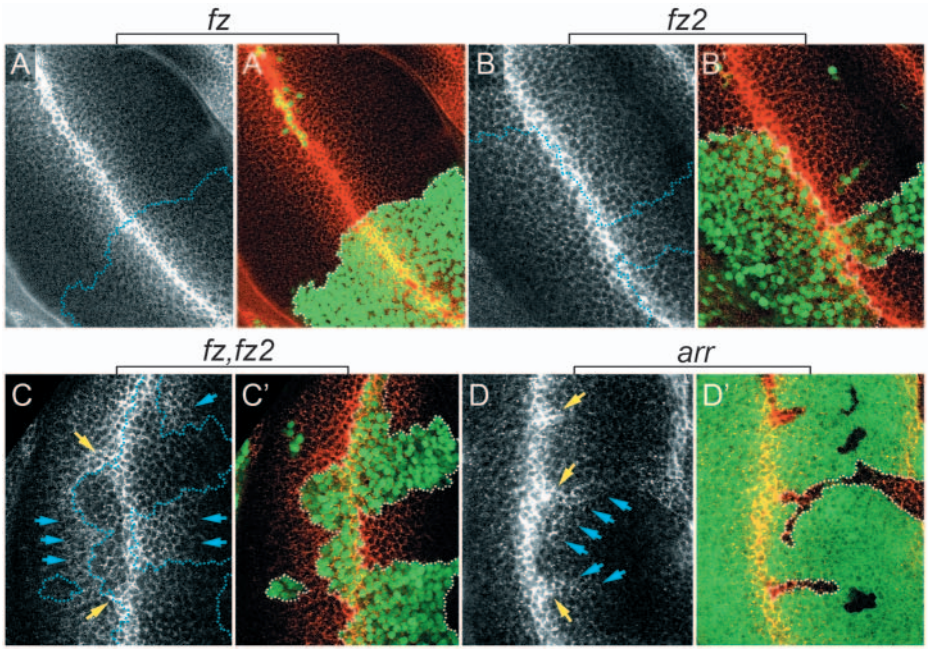

Fig. 4. The roles of Wg receptors in extracellular Wg distribution. Extracellular Wg staining in wing discs carrying mutant clones for *fz* (A,A′), *fz2* (B,B′), *fz-fz2* (C,C′) and *arr* (D,D′). Discs are oriented dorsal top-right, anterior top-left (A-B′), and dorsal right, anterior up (C-D′). The mutant clones are marked by the absence of GFP and outlined by broken lines, except in D. Extracellular Wg is not altered in mutant clones for *fz* or *fz2* alone. By contrast, it is increased in clones mutant for *fz-fz2* and *arr*. The yellow arrows indicate the presumptive ectopic Wg-expressing cells, and the blue arrows indicate the mutant cells accumulating Wg on the cell surface (C,D).

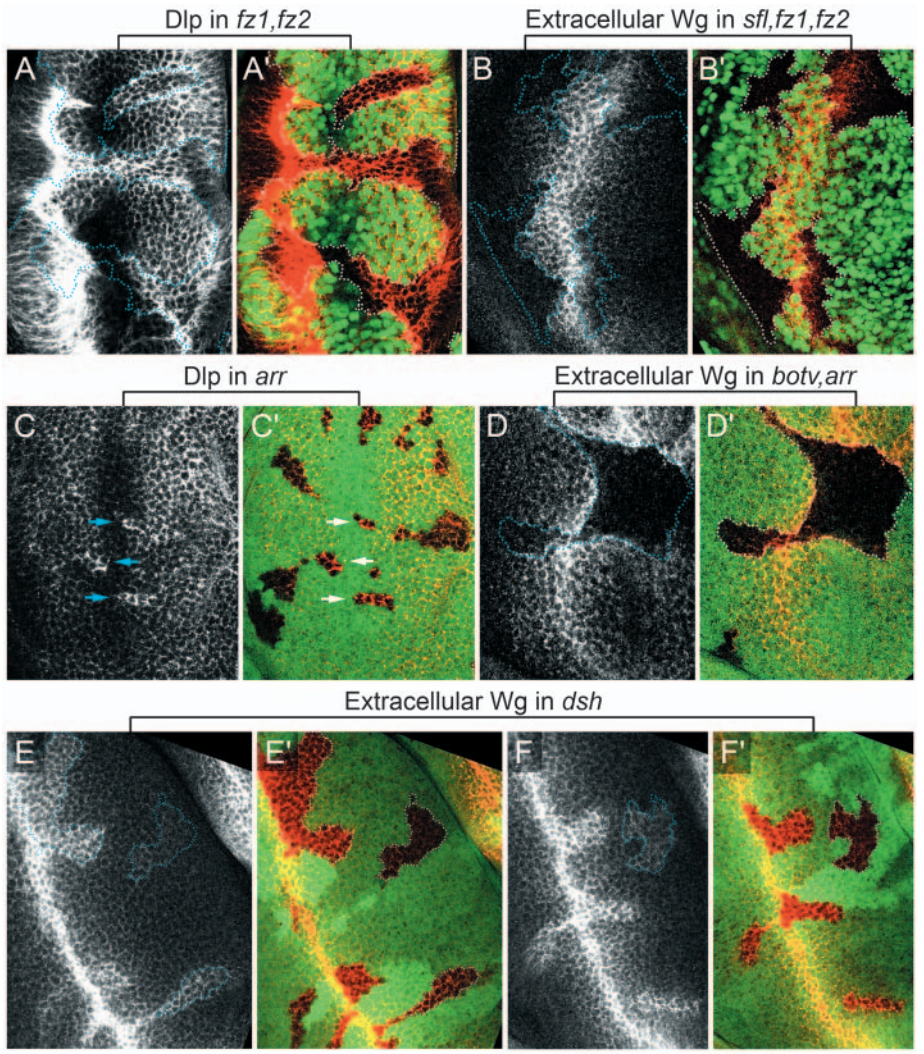

Fig. 5. Accumulated Wg in *fz-fz2* or *arr* clones are mainly resulted from upregulated Dlp. Extracellular Wg staining in wing discs carrying mutant clones of varying genotypes. Discs are oriented dorsal right, anterior up (A-D′), and dorsal top-right, anterior top-left (E-F′). The mutant clones are marked by the absence of GFP and outlined by broken lines except in C,C′. In F,F′, only the clone far away from DV boundary is outlined. (A,A′) A wing disc carrying clones mutant for *fz-fz2*. Dlp levels are enhanced in these clones. (B,B′) A wing disc carrying clones mutant for *sfl-fz-fz2*. Extracellular Wg levels are reduced in the clones. (C,C′) A wing disc carrying clones mutant for *arr*. Dlp levels are enhanced in the clones. (D,D′) A wing disc carrying clones mutant for *botv-arr*. Extracellular Wg is reduced in the clones. (E-F′) Wing discs carrying clones mutant for *dsh*. Extracellular Wg levels are increased in the clones. This effect can be clearly seen even the clones are located far away from Wg-expressing cells. Some of the mutant cells are located more than 20 cells away from the DV boundary.

virtually identical in the A and P compartments (Fig. 6A-A″) although the extracellular Wg level is marginally higher in the P compartment.

First, we ectopically expressed Dlp and Dally in the P compartment. Persistent induction of *UAS-dlp* by en^{Gal4} leads to a greatly reduced size in the P compartment of the wing disc (data not shown), presumably caused by interference of Wg signaling by Dlp. To overcome the deleterious effect of early induction of *UAS-dlp*, we used a temperature-sensitive allele of Gal80 ($Gal80^{ts}$) to keep Gal4 inactive until the late stage of larval development. $Gal80^{ts}$ functions as a repressor of Gal4 at the permissive temperature (19°C) but allows Gal4 to be active at the non-permissive temperature (30°C) (McGuire et al., 2003). When *UAS-dlp* is induced by hh^{Gal4} for 24 hours at the non-permissive temperature (30°C) in the presence of $Gal80^{ts}$, extracellular Wg is significantly increased on the surface of Dlp overexpressing cells and the visible range of the Wg gradient extends to the whole wing pouch along the AP axis (Fig. 6B,B′). The plot profile suggests that the Wg gradient becomes less steep and broader in the P compartment (Fig. 5B″). Importantly, the extracellular Wg level on the Wg-expressing cells is not significantly changed, suggesting that the high levels of Dlp at the DV boundary does not impede Wg movement. Similar results were obtained in the otherwise smaller posterior half of the disc when en^{Gal4} is used to induce the expression of *UAS-dlp* (data not shown). Consistent with previous results by others (Strigini and Cohen, 2000), overexpression of Dally by en^{Gal4} did not drastically alter extracellular Wg levels (Fig. 6C,C′). However, the plot profile shows that there is a mild increase in the Wg level in the region close to the DV boundary in the P compartment (Fig. 6C″), although this increase does not change the range and the steep shape of the Wg gradient significantly.

Second, we examined the ability of the Wg receptors (Fz and Fz2) in influencing the Wg gradient. Previous studies have shown that overexpression of Fz-GPI has no effect on the Wg gradient when examined by conventional staining (Rulifson et al., 2000). However, we found that the extracellular Wg levels are enhanced, and the Wg gradient becomes broader and flatter in the P compartment when overexpressing wild-type Fz (Fig. 7A-A″). The apparent difference between our observations and the previous work (Rulifson et al., 2000) most probably reflects a difference in the sensitivity of detection methods rather than in the affinity of two versions of Fz for Wg. Overexpression of Fz2, however, leads to increased Wg levels in the central zone of the P compartment (Fig. 7B,B′). The plot profile shows that the range of the Wg gradient is almost the same in the presence of a high level of Fz2 (Fig. 7B″), indicating that the primary effect of ectopic Fz2 is stabilizing Wg rather than enhancing Wg movement. We also found that Dlp levels are not altered in cells overexpressing Fz or Fz2 (data not shown), suggesting that the enhanced extracellular Wg levels are the direct result of overexpressing Fz and Fz2, both of which can, as suggested by biochemical studies, directly bind to Wg (Wu and Nusse, 2002).

Third, we examined the effect of Arr overexpression on extracellular Wg distribution. The extracellular Wg gradient is not significantly altered when GFP-Arr, a functional Arr molecule (see Materials and methods), is overexpressed by en^{Gal4} (Fig. 7C,C′). This result is consistent with the

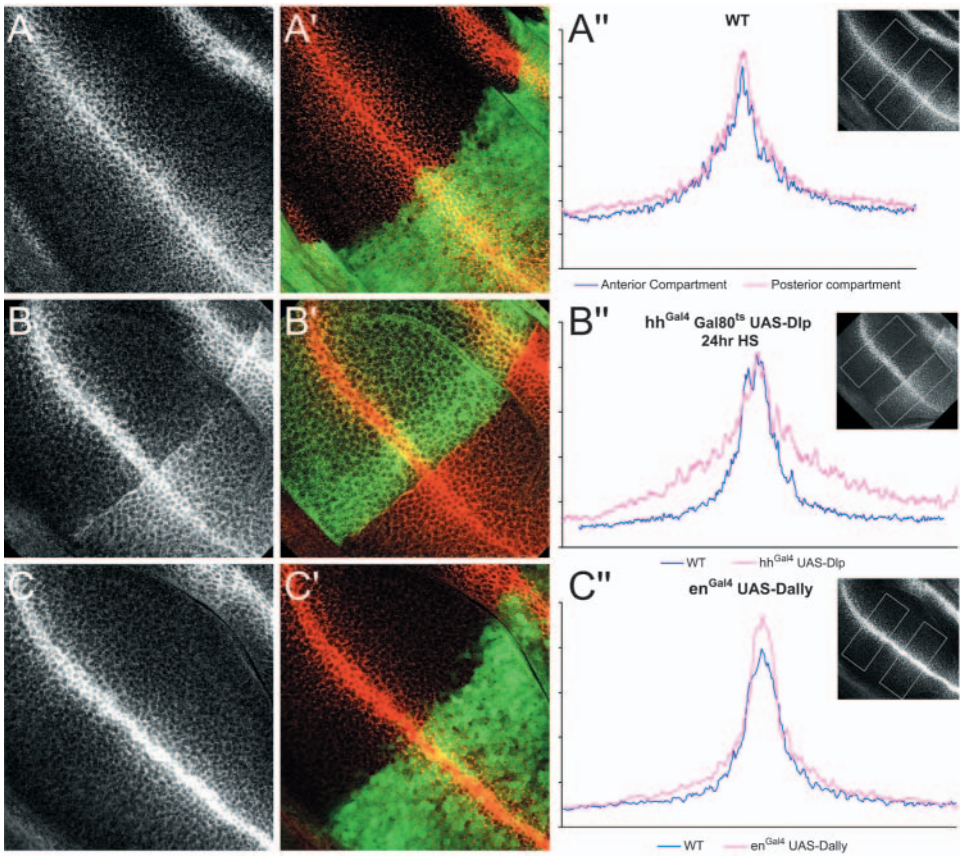

Fig. 6. The effects of overexpressing Dally and Dlp on extracellular Wg distribution. Extracellular Wg staining in a wild-type wing disc (A,A′), and discs overexpressing Dlp (B,B′) and Dally (C,C′) in the posterior compartment. The posterior compartment is marked by either GFP (green in A′ and C′) or the absence of Ci (green in B′). Signal profiles of extracellular Wg from selected areas in the anterior and posterior compartments of each wing disc are plotted and compared (A″,B″,C″). In the inset in each plot profile, the areas used for analysis are shown in box. The wing discs are oriented dorsal top-right, anterior top-left. The left-right axis of the plot profiles corresponds to the dorsoventral axis of the boxed regions.

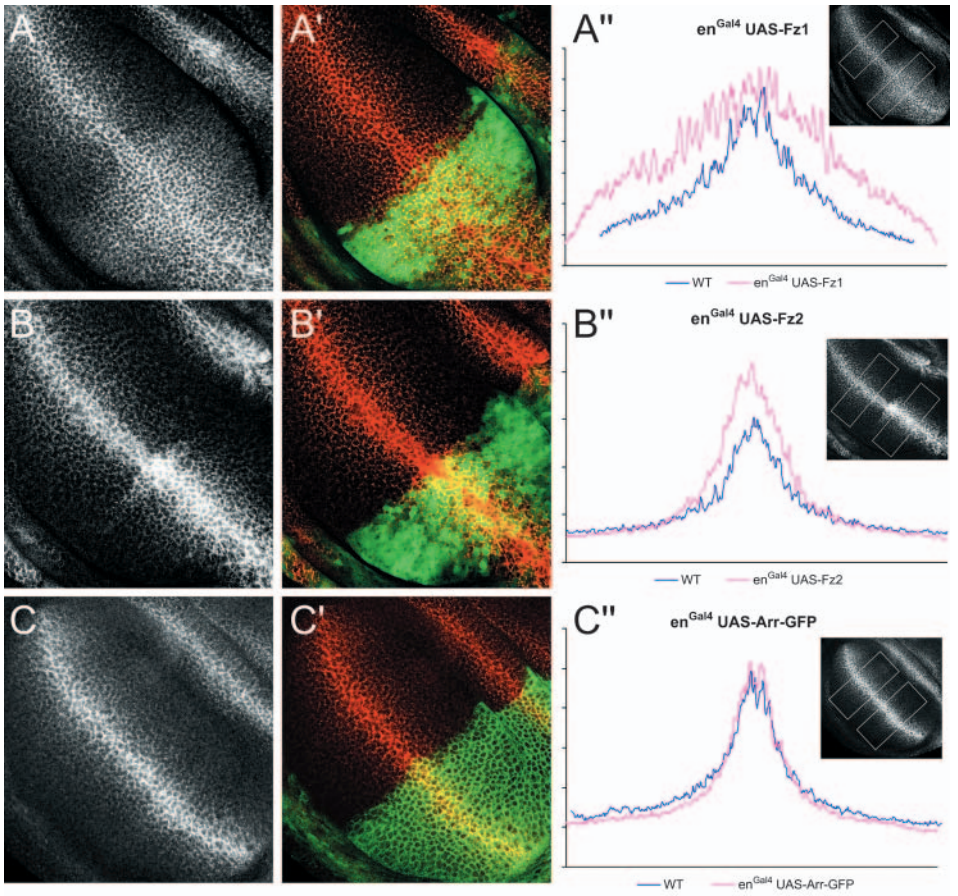

Fig. 7. The effects of overexpressing Fz, Fz2 and Arr on extracellular Wg distribution. Extracellular Wg staining in discs overexpressing Fz (A,A′), Fz2 (B,B′) and Arr-GFP (C,C′) in the posterior compartment. The posterior compartments are marked by GFP. The images and plot profiles are displayed as in Fig. 6.

biochemical study that Arr is not able to bind directly to Wg (Wu and Nusse, 2002). However, from the plot profile, we notice a slightly faster drop-off of the extracellular Wg gradient in the P compartment, which may be resulted from slightly enhanced Wg internalization/degradation in the presence of high levels of Arr.

Wg moves from cell to cell in a HSPG-dependent manner

Our recent studies demonstrated that Dally and Dlp are required for Hh and Dpp movement through a restricted diffusion mechanism (Belenkaya et al., 2004; Han et al., 2004b). We therefore examined whether Dally and Dlp control Wg movement through a similar mechanism. We generated narrow stripes of clones mutant for *sfl* or *dally-dlp* and asked whether Wg can move across these HSPG-deficient cells. Extracellular Wg levels are reduced within the *sfl* clones as well as in wild-type cells behind the clones (Fig. 8A-B′, shown by turquoise arrows), even though these clones are only one to two cell diameters wide. Similar results were obtained for *dally-dlp* clones (Fig. 8C-D′), suggesting that Wg movement is impeded by cells mutant for both *dally* and *dlp*. We also quantified this cell non-autonomous effect by generating profiles of Wg distribution in a region covering both the clones and wild-type areas behind the clones. The plots show that extracellular Wg is reduced to basal levels in the wild-type cells behind the *sfl* or *dally-dly* mutant clones (Fig. 8A″,B″,C″,D″). Collectively, these data argue that Wg movement is not by free diffusion, but rather is mediated by a restricted diffusion involving the HSPGs Dally and Dlp.

Discussion

The establishment of the long-range Wg gradient in the wing disc is a complex process involving multiple cell-surface molecules. We present evidence that the HSPGs Dally and Dlp are required for the formation of the Wg gradient. Surprisingly, although overexpression of Wg receptors (Fz and Fz2) can alter the Wg gradient, they are not essential for extracellular Wg gradient formation. We further demonstrate that Wg fails to move across *sfl*- or *dally-dlp*-deficient cells. These new findings argue that the HSPG Dally and Dly are the main factors for extracellular Wg gradient formation. Our results are consistent with a model in which the Wg morphogen moves along the plane of the disc epithelium through the HSPGs Dally and Dlp.

The glypicans Dally and Dlp in Wg gradient formation

One important finding in this work is that the glypicans Dally and Dlp are required for Wg gradient formation. Several recent studies have shown that extracellular Wg distribution is compromised in clones mutant for HS biosynthesis enzymes, including *sfl*, *slalom* and members of the *Drosophila* EXT gene (Baeg et al., 2001; Bornemann et al., 2004; Han et al., 2004a; Luders et al., 2003; Takei et al., 2004). However, it is unclear which HSPG cores are involved in this process. We show that Wg morphogen distribution is defective in either *dally* or *dlp* mutant clones. These new findings clearly establish the requirement of Dally and Dlp in Wg morphogen gradient formation. Thus, as in the case of Hh and Dpp (Belenkaya et al., 2004; Han et al., 2004b), the glypican members Dally and Dlp, rather than *Drosophila* syndecan or perlecan, are the main HSPGs involved in Wg gradient formation.

Interestingly, we found that Dally and Dlp differentially regulate the Wg extracellular gradient in distinct regions of the wing disc. Both Dally and Dlp are glypican members of HSPG family. One would expect that differences in the structure of Dally and Dlp, and their attached HS GAG chains may determine their abilities to interact with Wg, thereby leading to their specificities. This is probably one of the factors, as

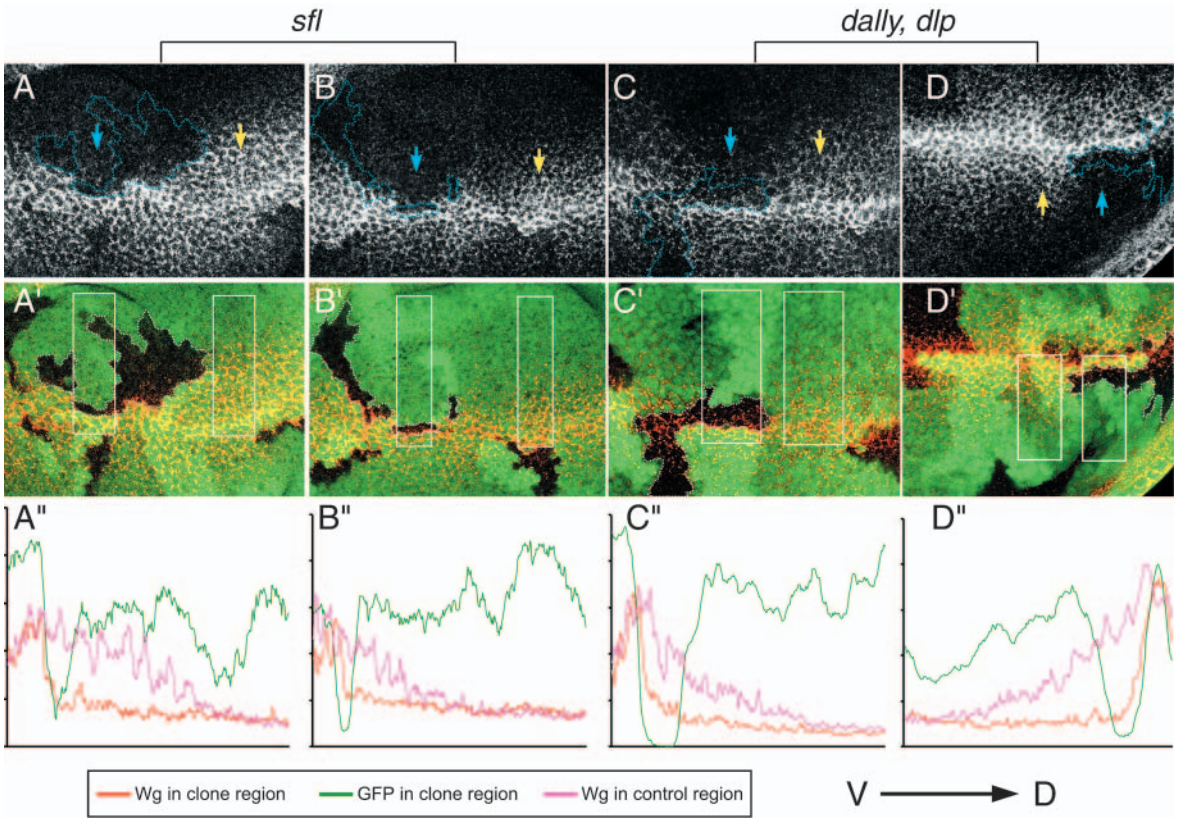

Fig. 8. Wg movement is impeded by HSPG-deficient cells. Extracellular Wg staining in discs bearing narrow stripes of clones mutant for *sfl* (A-B′) or *dally-dlp* (C-D′). All discs are oriented anterior left, dorsal up. The mutant clones are marked by the absence of GFP in the merged images, and outlined by broken lines. The extracellular Wg is reduced not only within these mutant clones, but also in the wild-type cells behind them (pointed by blue arrows). For comparison, the yellow arrows indicate wild-type cells at similar distances from the DV boundary. In each disc, Wg distribution is averaged and plotted (A″,B″,C″,D″) from an area including the narrow clone and the cells behind it and from a control area (boxed in the merged images). The colors of profiles are specified at the bottom of the figure. The left-right axis of the plot profiles corresponds to the ventrodorsal axis of the boxed regions. The drops of GFP intensity indicate the position of the mutant clones.

overexpression of Dally and Dlp has very different effects on extracellular Wg gradient. Consistent with our data in this work, previous studies have shown that Dlp is much more potent in accumulating Wg protein than Dally when overexpressed (Baeg et al., 2001; Giraldez et al., 2002). However, we have also found that the regional effects of Dally and Dlp on extracellular Wg gradient correspond well to their expression patterns. The regions with higher expression levels of Dally or Dlp have stronger extracellular Wg defects when Dally or Dlp is removed, respectively. Based on these data, we suggest that the differential roles of Dally and Dlp in extracellular Wg distribution are at least partially determined by their restricted expression.

What exact roles do Dally and Dlp play in shaping the extracellular Wg gradient? Our loss-of-function results suggest that removal of Dally or Dlp leads to reduced extracellular Wg levels on the cell membrane. Furthermore, extracellular Wg levels are reduced in wild-type cells behind *sfl* or dally-dlp clones (Fig. 8). These data suggest that the primary function of Dally and Dlp in Wg gradient formation is to maintain extracellular Wg proteins so that locally concentrated Wg proteins can further move to more distal cells through diffusion.

Roles of Dlp, Dally and Notum in modulating Wg signaling

Despite a positive role of Dlp in extracellular Wg distribution, surprisingly, Dlp negatively regulates Wg signaling at the DV boundary. However, ectopic Wg signaling at the DV boundary of the *dlp* mutant is considerably weak. This relatively weak effect is most probably due to the low level expression of Dlp, which is downregulated by Wg signaling. Our results are consistent with our previous observation that overexpression of Dlp in the wing disc leads to a blockage of Wg signaling (Baeg et al., 2001). Dlp may compete with Fz proteins for available Wg protein at the DV boundary, thereby inhibiting Wg signaling. However, the extract mechanism of Dlp-mediated Wg inhibition needs to be further determined.

Previous studies have identified Notum as a secreted inhibitor for Wg signaling (Gerlitz and Basler, 2002; Giraldez et al., 2002). Notum is expressed at the DV boundary and was proposed to downregulate Wg signaling by modulating Dlp activity (Giraldez et al., 2002). During the reviewing process of this manuscript, Cohen's (Kreuger et al., 2004) and Selleck's (Kirkpatrick et al., 2004) laboratories published their recent studies proposing that Notum negatively regulates Wg signaling by shedding of Dlp, which converts Dlp from a

membrane-tethered co-receptor to a secreted antagonist. Their conclusions are mainly based on two lines of experimental data. First, biochemical experiments clearly demonstrated that Notum can modify Dlp in a manner that resembles cleavage of the GPI anchor (Kreuger et al., 2004). Second, Kirkpatrick et al. showed that transheterozygous *dlp/notum* flies produced ectopic mechanosensory bristles which are not seen in $dlp^{+/-}$ or $notum^{+/-}$ alone, indicating that Dlp and Notum genetically collaborate in downregulating Wg signaling (Kirkpatrick et al., 2004).

However, on the basis of our data in this work, we suggest that Notum inhibit Wg signaling mainly by modifying Dally in the wing disc. First, genetic interaction data shown by Kirkpatrick et al. cannot distinguish whether Dlp and Notum work in the same pathway or in two independent pathways to downregulate Wg signaling at the DV boundary (Kirkpatrick et al., 2004). If Dlp is indeed the main substrate for Notum, we would expect that ectopic Wg signaling activity in *dlp-notum* should be similar to that in *dlp* mutant. However, our loss-of-function analysis demonstrates that ectopic Wg signaling in *dlp-notum* is similar to that in *notum* mutant, but much stronger than that in *dlp* mutant. However, *dally-notum* clones exhibits loss of Wg signaling activity, which is similar to *dally* mutant. Second, Dlp expression is strikingly repressed by Wg signaling and this reduction is independent of Notum. Low/absent expression of Dlp is not consistent with the view that Dlp is the main substrate for Notum. Finally, it is important to mention that Notum can reduce the amount of Dally when they are co-expressed in *Drosophila* S2 cells (Giraldez et al., 2002), suggesting that Notum can modify Dally as well. Although Notum can shed Dlp, whether shed Dlp acts as a Wg inhibitor need to be further determined (Kreuger et al., 2004). Therefore, further experiments are necessary to define the mechanism(s) of Notum-mediated Wg inhibition.

Roles of Fz, Fz2 and Arr in Wg gradient formation

One important finding of this study is that removal of the Wg receptors (Fz and Fz2) and the co-receptor Arr does not lead to a loss of extracellular Wg. Fz2 has been proposed to play a major role in Wg gradient formation in the wing disc by ectopic expression studies (Cadigan et al., 1998). Although several previous studies as well as our data in this work demonstrated the high capacity of Fz2 in stabilizing Wg (Cadigan, 2002; Lecourtois et al., 2001; Rulifson et al., 2000), our loss-of-function results clearly show that extracellular Wg levels were not reduced in clones mutant *fz2*. This is apparently not due to the overlapping function of Fz as the extracellular Wg level is enhanced, rather than reduced in the absence of both Fz and Fz2 functions. Our results argue that Fz2 is not essential for extracellular Wg gradient formation in vivo. It is important to note that in addition to Fz and Fz2, *Drosophila* Fz3 is also expressed in the wing disc and its expression is upregulated by Wg signaling (Sato et al., 1999). Although Fz3 has lower affinity than Fz2 in Wg binding and acts as an attenuator of Wg signaling (Sato et al., 1999; Wu and Nusse, 2002), its role in Wg distribution needs to be determined.

We further demonstrated that extracellular Wg is enhanced in cells mutant for *fz-fz2* or *arr*, suggesting that Wg receptors (Fz and Fz2) and Arr shape extracellular Wg gradient by downregulating extracellular Wg levels. Our data argue that this is mainly resulted from upregulation of Dlp. Consistent with this view, we show that the accumulated extracellular Wg can be eliminated by loss of HSPGs in *sfl-fz-fz2* or *arr-botv* mutant clones (Fig. 5). Importantly, we show that both extracellular Wg and Dlp levels are upregulated on the cell surface of clones mutant for *dsh*. These data provide compelling evidence that though a feedback mechanism, Wg signaling can control the Dlp levels to regulate the extracellular Wg gradient.

Another alternative possibility is that enhanced Wg levels in *fz-fz2* or *arr* clones may be caused by impaired Wg internalization. Although we observed significant amount of internalized Wg vesicles in *fz-fz2* or *arr* mutant clones (data not shown), we cannot rule out this possibility as a quantitative comparison of Wg internalization between wild-type cells and *fz-fz2* or *arr* mutant cells was difficult in our experimental settings. Furthermore, as mentioned above, Fz3 is expressed in the wing disc and its expression is upregulated by Wg signaling (Sato et al., 1999). It is possible that Fz3 may mediate the internalization of Wg in the absence of Fz and Fz2.

Molecular mechanisms of Wg movement

A previous study by Strigini and Cohen provided evidence that Wg morphogen movement is regulated by a diffusion

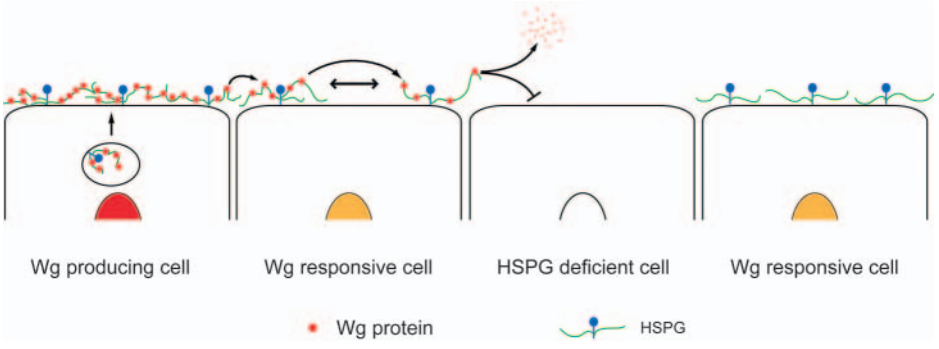

Fig. 9. A model of restricted Wg movement along cell surface. Secreted extracellular Wg is bound and stabilized by glypicans Dally and Dlp on the cell surface. The differential concentration of Wg from producing cells to receiving cells drives Wg diffusion along cell surface to more distal cells through disassociation/re-association with HSPGs. In the absence of Dally and Dlp, the Wg protein can no longer move further. It either stays on cell membrane or diffuses into disc lumen, or is degraded. The thin black arrows at the top of this diagram indicate the displacement of Wg from one GAG chain to another. The double-headed arrow indicates the lateral movement of glypicans on the cell membrane.

mechanism(s) in the wing disc (Strigini and Cohen, 2000). Does Wg diffuse freely in the extracellular matrix/space? In this work, we show that Wg fails to move across a strip of cells mutant for the HSPGs Dally and Dlp. This result suggests that Wg can not freely diffuse in the extracellular matrix. Instead, our findings are consistent with a model in which Wg movement is mediated by the HSPGs Dally and Dlp through a restricted diffusion along the cell surface (Fig. 9). Similar mechanisms have been proposed for Hh and Dpp (Belenkaya et al., 2004; Han et al., 2004b). In biological systems such as imaginal discs, the restraint of Wg spreading to the surface of the epithelial cell layer is important as the folding of imaginal discs, such as the leg disc, poses a problem if the Wg gradient formation were to occur out of the plane of the epithelial cell layer through free diffusion. In agreement with this view, our model proposes that Wg gradient formation depends on Wg movement through the cell surface of the disc epithelium.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/4/667/DC1

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