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The Wingless morphogen gradient is established by the cooperative action of Frizzled and Heparan Sulfate Proteoglycan receptors

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Abstract

We have examined the respective contribution of Heparan Sulfate Proteoglycans (HSPGs) and Frizzled (Fz) proteins in the establishment of the Wingless (Wg) morphogen gradient. From the analysis of mutant clones of *sulfateless/N-deacetylase-sulphotransferase* in the wing imaginal disc, we find that lack of Heparan Sulfate (HS) causes a dramatic reduction of both extracellular and intracellular Wg in receiving cells. Our studies, together with others [Kirkpatrick, C.A., Dimitroff, B.D., Rawson, J.M., Selleck, S.B., 2004. Spatial regulation of Wingless morphogen distribution and signalling by Dally-like protein. Dev. Cell (in press)], reveals that the Glypican molecule Dally-like Protein (Dlp) is associated with both negative and positive roles in Wg short- and long-range signaling, respectively. In addition, analyses of the two Fz proteins indicate that the Fz and DFz2 receptors, in addition to transducing the signal, modulate the slope of the Wg gradient by regulating the amount of extracellular Wg. Taken together, our analysis illustrates how the coordinated activities of HSPGs and Fz/DFz2 shape the Wg morphogen gradient.

Keywords: HSPG; Glypican; Wingless; Frizzled; Morphogen; *Drosophila*

Introduction

The organization of fields of cells is controlled by the action of 'form-giving' secreted molecules known as morphogens. Different levels of morphogen, which emanate from a localized source and diffuse across a field of cells, are thought to generate different patterns of transcriptional activity in responding cells, thus specifying differentiation programs that vary with distance from the morphogen-producing cells (reviewed in Seto et al., 2002; Tabata, 2001). Secreted signaling molecules of the Wnt/Wingless (Wg), Hedgehog (Hh), and Decapentaplegic (Dpp)/BMP/Nodal families have been shown to function as morphogens in many developing systems, both during embryonic development and organogenesis (reviewed in Roelink, 1995).

wg encodes secreted glycoproteins that regulate cell fates in many developmental processes. In the wing imaginal discs of late third instar larvae, *wg* RNA is expressed at the dorso-ventral (D/V) border, and the Wg protein acts up to 20–30 cell diameters away from its site of synthesis and triggers a transcriptional response of target genes in a concentration-dependent manner (Cadigan et al., 1998). Short-range Wg signaling induces the expression of the zinc finger transcription factor *senseless* (*sen*) in the sensory organ precursors (SOPs) along the presumptive wing margin (Nolo et al., 2000). Long-range Wg signaling controls the expression of *Distal-less* (*Dll*) within the wing blade (Neumann and Cohen, 1997; Zecca et al., 1996). Thus, Wg acts both as a short- and a long-range inducer

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(Neumann and Cohen, 1997; Zecca et al., 1996). Wg that originates from the D/V border is detected in an irregular pattern of puncta in its receiving cells, and the intensity and number of these puncta is graded and decreases from the source of Wg. A number of studies have implicated two seven-transmembrane receptors Frizzled (Fz) and DFrizzled 2 (DFz2), and the two glypican molecules Dally and Dally-like protein (Dlp) in efficient Wg signal transduction and in regulating the formation of the Wg morphogen gradient (Baeg et al., 2001; Bhanot et al., 1996; Cadigan et al., 1998; Kennerdell and Carthew, 1998; Lin and Perrimon, 1999; Muller et al., 1999; Perrimon and Haecker, 2004; Rulifson et al., 2000; Strigini and Cohen, 2000; Tsuda et al., 1999). However, the exact mechanism by which the Wg gradient forms and is maintained is still not well understood.

Classic cell biology points to the central role of receptors in regulating ligand endocytic trafficking and morphogen movement (reviewed in Vincent and Dubois, 2002). DFz2 has been shown to bind Wg with high affinity and its expression is down-regulated in response to Wg signaling (Cadigan et al., 1998). Importantly, ectopic expression of *DFz2* throughout the wing was found to cause an expansion of Wg-target gene expression resulting in the formation of ectopic hairs on the wing surface. These data were interpreted to indicate that DFz2 broadens the range of Wg action by protecting the ligand from degradation. Hence, Wg-mediated repression of *DFz2* expression appears to be crucial for the normal shape of the Wg morphogen gradient to create a gradual decrease in Wg concentration (Cadigan et al., 1998). The role of DFz2 in broadening the range of the morphogen is in contrast to other cases of ligand–receptor relationships where the receptor limits diffusion of the ligand. For example, in the context of Hh signaling, overexpression of the Hh receptor Patched, leads to a reduction of Hh movement, whereas removal of Patched activity in Hh-receiving cells leads to an expansion of Hh movement across the mutant clone (Bellaiche et al., 1998; Chen and Struhl, 1996). In embryonic tissues, Fz proteins have also been proposed to modulate the slope of the Wg gradient. However, in this case, they appear to trap and subsequently degrade the ligand because overexpression of a dominant-negative form of *DFz2* (Δ*DFz2-GPI*) driven by *en-Gal4* in embryonic tissue prevents Wg decays within the *en* domain. Interestingly, in the embryo, Fz proteins do not appear to be required for Wg movement because Wg puncta were found to distribute normally in *fz DFz2* mutant embryos (Chen and Struhl, 1999; Muller et al., 1999).

HSPGs are abundant cell surface molecules that are part of the extracellular matrix. A number of genes involved in HS GAG (heparan sulfate glycosaminoglycan) biosynthesis and modification have been implicated in Wg signaling (reviewed in Baeg and Perrimon, 2000; Cumberledge and Reichsman, 1997; Nybakken and Perrimon, 2002; Perrimon and Bernfield, 2000; Selleck, 2001). These include *sugarless* (*sgl*; UDP-glucose dehydrogenase; Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997), *fringe connection* (*frc*; UDP glucose transport; Goto et al., 2001; Selva et al., 2001), and *sulfateless* (*sfl*; *N*-deacetylase/*N*-sulphotransferase; Lin and Perrimon, 1999). Consistent with these results, the *Drosophila* glypican HSPG core proteins, Dally and Dlp, have been shown to serve as substrates for these HS biosynthetic enzymes to yield HSPGs that bind and stabilize Wg at the cell surface (Baeg et al., 2001; Lin and Perrimon, 1999; Selva et al., 2001; Strigini and Cohen, 2000). Altogether, these observations suggest that HSPGs are involved in Wg signaling presumably by regulating distribution of the ligand throughout tissues. Despite these studies, a number of issues regarding the role of HSPGs have not yet been resolved. First, it is not clear whether HSPGs are required in Wg-sending and/or -receiving cells. Second, the respective function of HSPGs and Fz/DFz2 receptors in Wg distribution and gradient formation is not understood. Here, we address how the coordinated activities of HSPGs and Fz/DFz2 shape the Wg morphogen gradient.

Materials and methods

Fly stocks

The following stocks were used: *UAS-Notum-GT* (gift from S. Cohen; Giraldez et al., 2002), *UAS-DFz2* (Cadigan et al., 1998), *UAS-dlp* (Baeg et al., 2001), *UAS-gfp-dlp* (this study), *C96-Gal4* on the third chromosome (Gusfafson and Boulianne, 1996), *ap-Gal4* on the second chromosome (Calleja et al., 1996), *wg-lacZ* (Kassis et al., 1992), *w; $sfl^{l(3)03844}$ FRT^{2A}/TM6C, Sb, Tb* and *w; sfl^{9B4}/TM6B, Tb* (Lin and Perrimon, 1999), *y w hs-flp; $M(3)^{i55}$ hs-GFP FRT^{2A}/TM6B, Tb* and *hs-flp; fz^{H51} $fz2^{C1}$ ri FRT^{2A}/TM2* (a gift from G. Struhl; Chen and Struhl, 1999), *Act> CD2>Gal4; hs-flp MKRS/TM6B, Tb* (Pignoni and Zipurski, 1997).

To analyze the role of the Fz receptors in Wg signaling, we generated clones that are doubly mutants for fz^{H5} and $fz2^{C1}$. fz^{H51} is associated with a single base change in the *fz* gene. This mutation creates a stop codon at tryptophan 500 and is a phenotypic null allele (Jones et al., 1996). $fz2^{C1}$ is associated with a single base change in the *Dfz2* gene. This mutation creates a stop codon located at the junction between the coding sequence of the amino-terminal extracellular domain and the remainder of the protein. When the $fz2^{C1}$ mutant protein was overexpressed in the wing imaginal disc, no effect on the distribution or signaling activity of Wg was observed, indicating that $fz2^{c1}$ is an amorphic mutation (Chen and Struhl, 1999). Thus, although both the *fz* and *DFz2* mutations that we used in our analysis are genetically null alleles, it remains a possibility that they do not correspond to protein null alleles.

UAS construct

pBS(KS)-gfp-dlp was generated by inserting the PCR amplifiedGFPfragment (5′agcatatgtagtgagcaagggcgaggagct, 3′tccatatggcttgtacagctcgtccat) from pEGFP-N1(Clontech) at a unique *Nde*I site in *pBS(KS)-dlp* (Baeg et al., 2001). *UAS-gfp-dlp* was created by cloning the full-length fragment (*Eco*RI–*Xho*I) from *pBS(KS)-gfp-dlp* into pUAST (Brand and Perrimon, 1993).

Clonal analysis

To generate Flip-out clones second instar larvae (*Act> CD2>Gal4/+; hs-flp MKRS/UAS-Notum-GT, Act> CD2>Gal4/+; UAS-DFz2/+; hs-flp MKRS/+*) were treated by heat-shock (120 min at 37°C). Wing discs in late third instar larvae were dissected and stained. *sfl* mutant clones were induced by heat shock (45 min at 37°C) during the second instar larvae (*y w hs-flp/ y w hs-flp; M(3)^{i55} hs-GFP FRT2A/TM6B* crossed to *w/Y; sfl$^{l(3)03844}$ FRT^{2A}/TM6C*). Wing discs from non-Tubby larvae were dissected and stained. *Fz DFz2* double-mutant clones were induced by either heat shock (45 min at 37°C) in early–mid third instar larvae *y w hs-flp/y w hs-flp; M(3)^{i55} hs-GFP FRT^{2A}/TM6C* crossed to *hs-flp/Y; fz^{H51} fz2^{C1} ri FRT^{2A}/TM2* or heat shock (1 h at 37°C) in second instar larvae *y w hs-flp/y w hs-flp; ubi-GFP FRT^{2A}/TM3* crossed to *hs-flp/Y; fz^{H51} fz2^{C1} ri FRT^{2A}/TM2*, and then the discs from late third instars were stained.

Texas-red dextran and antibody labeling

For Texas-red dextran labeling, discs from third instar larvae were incubated in 0.25 mM Texas-red dextran in M3 medium for 10 min at room temperature, washed five times for 2 min with ice-cold M3 medium, incubated for 20 min at RT (chase) and then fixed in 4% formaldehyde at room temperature for 20 min (Entchev et al., 2000). For antibody stains, the following primary antisera were used: rabbit anti-β-gal (Cappel) at 1:2000 dilution; mouse anti-Wg (4D4; Hybridoma Bank, Brook and Cohen, 1996) at 1:10 dilution; rabbit anti-Notum (gift from S. Cohen, Giraldez et al., 2002) at 1:25 dilution, rabbit anti-DFz2 (gift from S. Cumberledge, unpublished) at 1:1000 dilution, rabbit anti-Dlp (gift from S. Baumgartner; Baeg et al., 2001) at 1:50 dilution; guinea pig anti-Sen (gift from H. Bellen, Nolo et al., 2000) at 1:1000 dilution. Extracellular Wg or Dlp in wing discs were detected by incubation with anti-Wg or -Dlp before fixation or detergent treatment (for details, see Strigini and Cohen, 2000). Fluorescent secondary antibodies from Jackson ImmunoResearch Labs were used at 1:200 dilution and AlexaFlours from Molecular Probes at 1:500 then incubated at room temperature for 2 h. Discs were mounted in Vectashield mounting media and inspected using a Leica TCS-NT or Zeiss LSM510 confocal microscope.

RNAi experiment and cell-based assay

Embryo-derived S2R+ cells (Yanagawa et al., 1998) were used in the RNAi experiment. Transfections were performed in duplicate in 384-well plates using Effectene transfection reagent (Qiagen). The ratio of Luciferase reporter (Top12X-HS-Luciferase; R. Dasgupta and N. Perrimon, in preparation), normalization vector (Renilla luciferase, PolII-RLuc) and Inducer (pMK33-Wg, Yanagawa et al., 1998) DNA was 1:1:2 with 100 ng of total DNA added per well. We used an inducible metallothionein promoter to drive *wg* expression (pMK33-Wg). dsRNAs were synthesized using in vitro transcription from PCR product templates, which have T7 polymerase binding sites as linkers (as described in Boutros et al., 2004). Eighty nanograms of dsRNA was added to each transfection reaction along with the total DNA.

The following dsRNAs were used: (1) Dlp; the primers used to amplify PCR product used for in vitro transcription to generate the dsRNA were, forward primer: ATGCTACATCAGCAGCAAC, reverse primer: ACTGGGTTTTTGGGGAATTTC; (2) Arm; forward primer: TGGTGGATGCAATAGCTTTAC, reverse primer: GACTACGAGAAGCTTCTGT; (3) Daxin; forward primer: CTCTACATCCAGCAGATGTC, reverse primer: TCGGATTTCCAGTCTTCTTTT.

Results and discussion

Heparan sulfate and Wg gradient formation

Sfl encodes a homolog of the Golgi enzyme HS *N*-deacetylase/*N*-sulfotransferase that is required for the modification of HS (Lin and Perrimon, 1999; Toyoda et al., 2000). We wanted to determine whether the retention of Wg at the cell surface involves HSPGs in receiving cells, since it has been proposed that HSPGs were unlikely to be required in Wg-receiving cells (Pfeiffer et al., 2002). In the wing blade, Wg, originating from the D/V border, is detected in an irregular pattern of puncta in receiving cells (Figs. 1A,B), which corresponds to the internalized Wg protein (Strigini and Cohen, 2000). The intensity and number of puncta decreases from the source of Wg. Furthermore, using the extracellular labeling method of Strigini and Cohen (2000), a gradient of Wg protein that appears broader, shallower, and with less puncta is observed (Fig. 1C). Both in *sfl* mutant wing discs and in large *sfl* mutant clones, a striking decrease in the number of Wg puncta was observed (Figs. 1E,G,H,I). This decrease was not due to a change in *wg* transcription because it was not affected in *sfl* mutant cells (Fig. 1D). Further, lack of Sfl activity did not appear to disrupt the overall amount of Wg produced by *wg*-expressing cells (Fig. 1E) but was associated with a dramatic decrease in extracellular Wg (Fig. 1F). These results suggest that

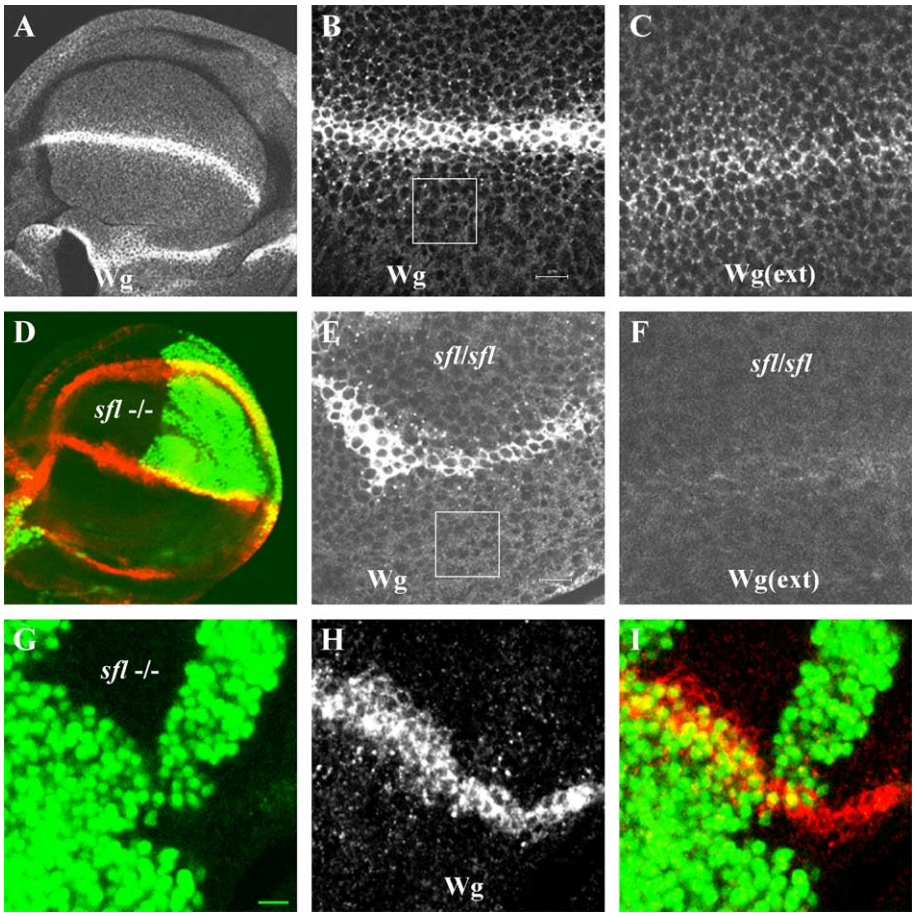

Fig. 1. Wg distribution in cells that lack *sfl* activity. (A–C) Wing discs labeled with mouse anti-Wg antibody using conventional (A, B) and extracellular (ext) labeling protocol (C). (D) Expression pattern of *wg-lacZ* (red) in *sfl* mutant clones (*sfl*−/−). *wg* transcription is not affected in *sfl* mutant clones that were detected by the absence of GFP. Intracellular (E) and extracellular (F) Wg was visualized using different labeling methods. (E) No alteration in the expression of intracellular Wg proteins in *sfl* ^{p1610}/*sfl*^{9B4} mutant wing disc was observed (compare to WT in B). However, a dramatic decrease of extracellular (ext) Wg proteins is observed in *sfl* ^{p1610}/*sfl*^{9B4} mutant disc (F) when compared to those in WT (C). Interestingly, *sfl* mutant wing discs show a decrease in the number of Wg puncta in receiving cells (E), which corresponds to the internalized Wg protein. The mean pixel values within a 20 × 20 μm box were averaged from three identically processed *sfl* (E) and WT (B) wing imaginal discs. Boxes were placed on the ventral side of the disc 3 cell diameters away from Wg expressing cells at the D/V boundary. The mean pixel values were 27.3 (±4.5) for *sfl* and 42.3 (±6) for WT. These results suggest that HSPGs are required for sequestering extracellular Wg in receiving cells. (G–I) Distribution of intracellular Wg (gray or red) in *sfl* mutant clones, which are detected by the absence of GFP. In *sfl* mutant clones, less Wg puncta are detected when compared to those in neighboring WT cells, suggesting that HSPGs are required in receiving cells to trap extracellular Wg. Scale bars: 5 μm (in E for E, F); 10 μm (in G for G–I).

HSPGs are required for sequestering extracellular Wg in receiving cells.

To gain further insights into the role of HSPGs in receiving cells to shape the Wg gradient, we examined the distribution of Wg in patches of WT cells located within a large *sfl* mutant territory. In such cases, we could detect bright spots of Wg within the patch of WT cells (Figs. 2A–C, red circle in B), indicating that *sfl*-expressing cells are able to sequester extracellular Wg, unlike neighboring cells that lack *sfl*. This result was consistent through an analysis of more than 10 clones. Further, we generated clones of cells that overexpress *Notum-GT* (Golgi-tethered), which acts cell autonomously in receiving cells. Previously, Giraldez et al. (2002) showed that *Notum*, which encodes a member of the α/β-hydrolase superfamily, antagonizes Wg signaling and these authors proposed that it acts by altering the ability of the cell surface glypican molecules Dally and Dlp to stabilize extracellular Wg. Consistent with the conclusion that HSPGs are required in receiving cells to capture extracellular Wg, we detected a decrease in the formation of Wg puncta in cells overexpressing *Notum-GT* (Figs. 2D–F). These results are consistent with at least two nonexclusive models. First, HSPGs could be required for Wg stability and/or trapping of Wg at the cell surface such that it does not diffuse away. Second, HSPGs could be involved in promoting Wg movement throughout tissues. The role of HSPGs in sequestering and/or stabilizing the ligand is supported by the previous observations that overexpression of either Dlp or Dally results in the accumulation of extracellular Wg (Baeg et al., 2001; Giraldez et al., 2002; Strigini and Cohen, 2000).

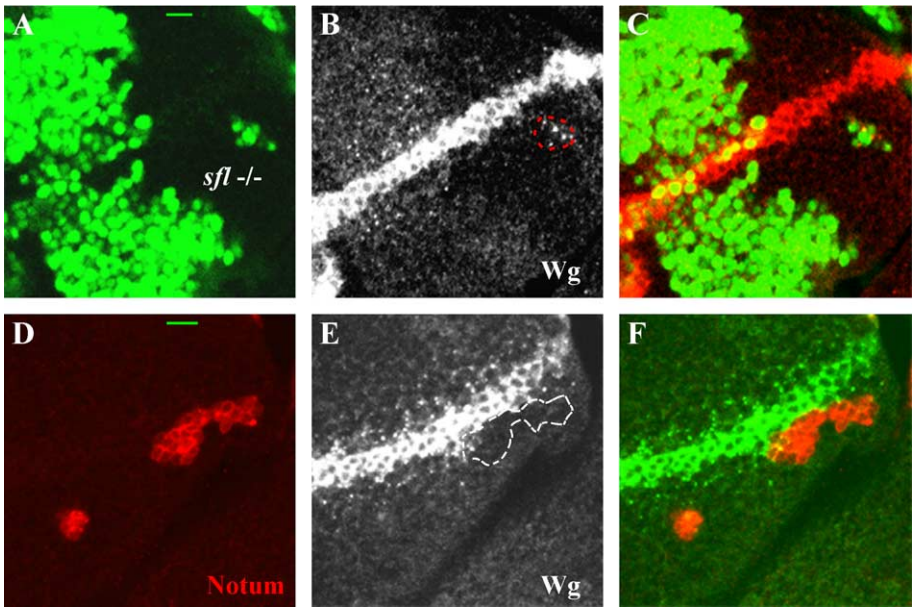

Fig. 2. The activity of HSPGs is essential for the binding of Wg to receiving cells. (A–F) Wg expression is visualized by the conventional labeling method. (A, B, C) Distribution of Wg (gray or red) in *sfl* mutant clones, which are detected by the absence of GFP. Consistent with the observation that HSPGs are required in receiving cells to sequester extracellular Wg, bright spots of Wg are detected in a patch of WT cells (marked by the red dotted line in B) within a large *sfl* mutant territory, although less puncta is observed in neighboring *sfl* mutant cells. (D–F) Cells overexpressing the Golgi-tethered form of Notum (Notum-GT) in receiving cells have less Wg puncta (gray or green) when compared to those in neighboring WT cells. The cells overexpressing *Notum-GT* are detected using the anti-Notum antibody (red) and indicated by the white dotted line in E. Scale bars: 10 μm (in A for A–C); 10 μm (in D for D–F).

Because HSPGs have been implicated in endocytosis of ligands such as FGF (Gleizes et al., 1995), possibly HSPGs also play a role in Wg internalization. A role for HSPG in Wg endocytosis would be consistent with the absence of puncta in *sfl* clones, and also the observed accumulation of extracellular Wg following overexpression of Dlp-HA (Giraldez et al., 2002). However, much of Wg proteins appeared in intracellular vesicles, instead of outlining the cell surface, in discs overexpressing both Dlp-HA and Notum. If HSPGs were directly involved in Wg internalization, we would have expected to detect fewer intracellular vesicles in discs overexpressing Dlp-HA and Notum since Notum acts to decease the affinity of Dlp for Wg. Furthermore, if the primary function of HSPGs were to internalize Wg, then we would expect to see extracellular Wg accumulation in cells lacking HSPGs activity, which is not the case (Fig. 1F). However, our data does not rule out the possibility that HSPGs play a direct role in Wg endocytosis, and, thus, further analysis will be required to clarify this issue. Taken together, our results suggest that the primary role of HSPGs is to trap and/or stabilize extracellular Wg in receiving cells where it is then able to interact with its signaling receptor as well as other factors that are responsible for its internalization, and thus contributes to shaping the Wg gradient.

Dlp distribution in the wing imaginal disc

Previous ectopic expression studies have shown that Dlp can trap extracellular Wg and prevent activation of the Wg signaling pathway (Baeg et al., 2001, Giraldez et al., 2002). Because Dlp appears to be a major HSPG required to regulate Wg signaling, we examined its endogenous distribution in the wing imaginal disc using a polyclonal antibody against Dlp and a staining method that primarily detects extracellular proteins (Strigini and Cohen, 2000). The specificity of the Dlp antibody was confirmed by misexpressing *dlp* using the *ap-Gal4* driver (Fig. 3C). In the third instar wing imaginal disc, Dlp was detected throughout the disc; however, a significant decrease in the level of Dlp was detectable at the D/V border (arrow in Fig. 3D). This domain of low Dlp expression correlates with the region where high level of Wg signaling is required to induce the expression of short-range target genes. We note that since *dlp* mRNA expression is uniform throughout the disc (X. Lin, personal communication), the down-regulation of Dlp at the D/V border must occur post-translationally. Interestingly, an optical cross section of the disc revealed that endogenous Dlp localizes mostly on the basolateral surface of the cell (Fig. 3E) where extracellular Wg is detected (Strigini and Cohen, 2000). The subcellular localization of Dlp protein was also examined using a *GFP-dlp* expressed under the control of a *Gal4* driver. Consistent with the Dlp antibody result, we found that GFP-Dlp localizes predominantly to the basolateral membrane (Fig. 3F). Altogether, these observations suggest that Dlp can bind to extracellular Wg and that Dlp levels need to be reduced for high-level Wg activity in cells near the D/V boundary.

Dlp acts as both a negative and positive regulator of Wg signaling in tissue culture cells

In a genome-wide RNAi screen in S2R+ cells (R. Dasgupta and N. Perrimon, in preparation) to identify genes that either up- or down-regulate Wg signaling, we identified Dlp as both a negative and positive regulator of Wg signaling under stimulated and nonstimulated conditions, respectively. The cell-based assay we devised consists of the activation of a Tcf/Arm-dependent Wg-reporter gene upon induction of S2R+ cells by expressing a *wg* cDNA by transient transfection (Fig. 4). The activity of the Wg pathway and the effect of the addition of various dsRNAs on the pathway were assayed by monitoring Luciferase-reporter activity using a luminescence-based plate reader. Using this assay, the addition of dsRNAs of positive transducers of Wg signaling, such as *arm*, decrease the Top12X-HS-Luciferase reporter activity, while dsRNAs to negative Wg regulators, such as *Daxin*, increase its activity. Interestingly, under condition of Wg induction (Fig. 4A), we found that *dlp* acts as a negative regulator of Wg signaling, as *dlp dsRNA* led to a twofold increase in luciferase activity. This increase is significant as it is comparable to that of *daxin dsRNA* (Fig. 4A). In the absence of Wg induction, we found that *dlp* positively regulates Wg signaling, as *dlp dsRNA* led to a fivefold decrease in luciferase activity (Fig. 4B), a decrease that is similar to that observed by the addition of *arm dsRNA.* These results suggest Dlp acts as a positive regulator of the Wg pathway when Wg level is low and negatively influences signaling when Wg is abundant. These results are consistent with in vivo results from the Selleck laboratory that demonstrated that Dlp has both a positive and negative role in Wg signaling (Kirkpatrick et al., 2004). Our observations in S2R+ are consistent with our hypothesis that low Dlp levels at the D/V boundary supports high-level Wg signaling, while further away from the D/V boundary where the Wg concentration is lower, Dlp positively influences Wg signaling. Overall, the result from the S2R+ RNAi experiments indicates that (1) Dlp is not an essential component of the Wg signal transduction pathway; and (2) Dlp can either have a negative or positive impact on Wg signaling depending on the level of Wg available. The negative effect of Dlp is consistent with previous in vivo studies that showed ectopic Dlp expression can trap extracellular Wg and prevent activation of the Wg signaling pathway (Baeg et al., 2001, Giraldez et al., 2002). Therefore, a reduction of Dlp levels at the D/V border would be expected to contribute to high-level Wg signaling. The positive effect of Dlp in Wg signaling needs to be understood in the context of the previous findings that loss of HSPG activity results in *wg* loss-of-function phenotypes, as shown by a decrease in *dll* expression in clones mutant for enzymes involved in GAG biosynthesis (Takei et al., 2004). One attractive model is that Dlp would act as a co-receptor that traps/stabilizes extracellular Wg and facilitates its association with the signal transducing Fz receptors in

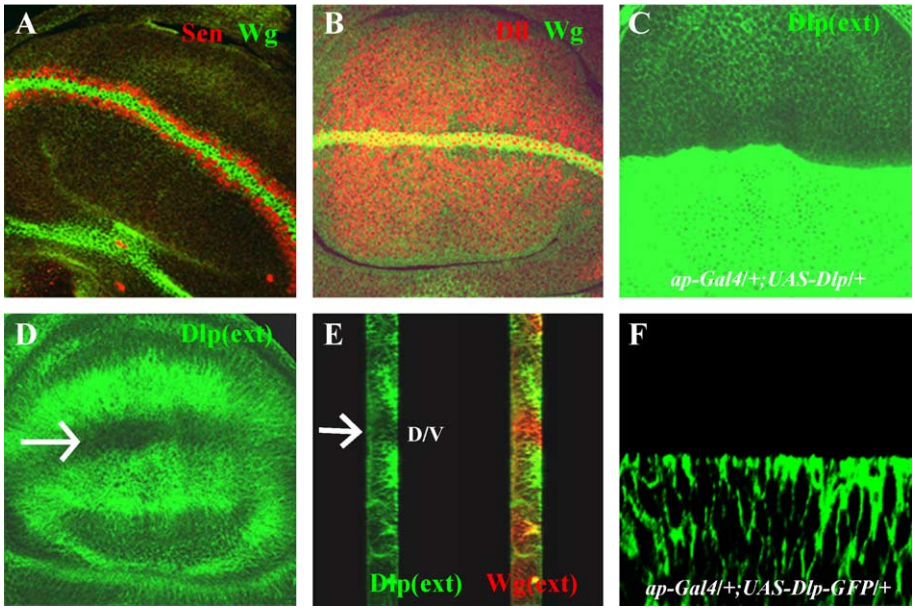

Fig. 3. The relationship between Wg activity and Dlp distribution in the wing imaginal disc. (A, B) Expression pattern of Wg short- and long-range target genes, and Wg (green) is visualized using conventional staining protocol. (A) Short-range Wg signaling induces the expression of the *senseless* (*sen*, red), which is expressed in the sensory organ precursors (SOPs) along the presumptive wing margin. (B) Long-range Wg signaling controls the expression of *Distal-less* (*Dll*, red) within the wing blade. (C–F) Distribution of Dlp. (C) The wing disc (*ap-Gal4/+*; *UAS-dlp/+*) is labeled for extracellular Dlp by the extracellular labeling method using the anti-Dlp antibody. Note the high expression of Dlp in the dorsal compartment, illustrating the specificity of the anti-Dlp antibody. (D) Endogenous extracellular Dlp is visualized. In these experiments, Dlp is found ubiquitously throughout the wing pouch, but low level of Dlp is detected near the Wg source (arrow). (E) Optical cross section of the wing pouch clearly shows decreased level of Dlp near the source of Wg production (arrow). (F) Optical cross section at the dorsal compartment of the disc overexpressing *GFP-dlp* driven by *ap-Gal4*. Note that GFP-Dlp localizes predominantly to the basolateral membrane.

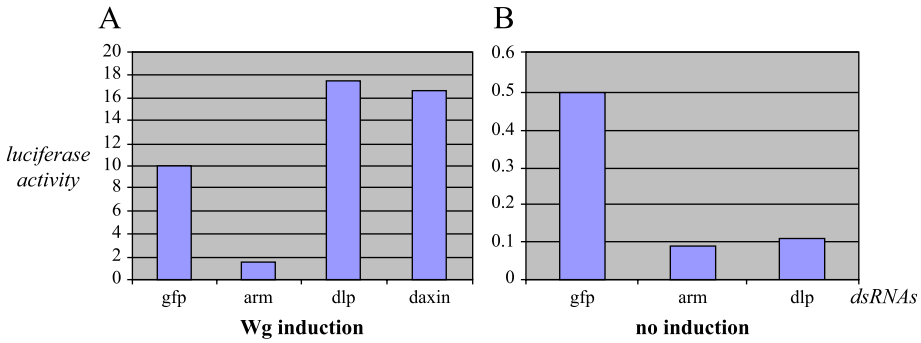

Fig. 4. Dlp has a negative and positive role in Wg signaling in S2R+ cells. S2R+ cells were transfected with the Top12X-HS-Luciferase reporter gene and the Renilla normalization vector with (A) and without (B) the Wg induction (see Materials and methods). (A) In the presence Wg induction, the first column represents luciferase activity in the presence of *GFP* dsRNA. Addition of *arm* dsRNA led to an eightfold reduction in reporter activity, whereas addition of *daxin* dsRNA resulted in a twofold increase in reporter activity. Interestingly, addition of *dlp* dsRNA led to a twofold increase in reporter activity. This increase is significant as it is comparable to that of *daxin* dsRNA. (B) In the absence of Wg induction, Dlp positively regulates Wg signaling, as *dlp dsRNA* led to a fivefold decrease in luciferase activity. The effect of *dlp* dsRNA on Wg activity is similar to that of *arm* dsRNA. Taken together, these results suggest Dlp acts as a positive regulator of the Wg pathway when Wg level is low and negatively influences signaling when Wg is abundant. We note that a fivefold increase in Wg signaling was observed when *dlp dsRNA* was added to clone 8 cells under Wg induction. However, these cells were inappropriate to evaluate the effect of *dlp dsRNA* without induction due to low basal Wg activity (data not shown).

cells located at a distance from the D/V boundary where low level of Wg is available. Finally, our observations are consistent with the recent study of Kirkpatrick et al. (2004) who showed that (1) ectopic activation of Wg signaling at the wing margin occurred in *dlp* mutant tissues; and (2) a cell autonomous reduction in Wg signaling in *dlp* clones located distal to the Wg-producing cells.

Fz-DFz2 receptors and Wg movement

The distribution of Dlp protein is reminiscent to the down-regulation of *Dfz2* transcription near the D/V border. Previously, Cadigan et al. (1998) showed that Wg-mediated repression of *DFz2* expression affects the shape of the Wg gradient, resulting in a gradual decrease in Wg concentration. Because our results indicate that HSPGs affect Wg distribution, we reexamined the function of the two seven transmembrane Wg receptors, Fz and DFz2, to evaluate how the signal transducing receptors cooperate with HSPGs in shaping the Wg gradient.

To determine the role of Fz and DFz2 in Wg movement, we examined the distribution of Wg in *fz DFz2* double-mutant clones. In these clones, we first observed an expansion of *wg* expression, which is consistent with the previously described Wg "self-refinement" process, by which Wg signaling represses *wg* expression in cells adjacent to *wg*-expressing cells (Rulifson et al., 1996). Unexpectedly, we also found that within these clones, Wg puncta are still present (Figs. 5A–C), indicating that Fz/DFz2 receptor activities are not required for Wg spreading.

To determine whether Wg is present in endosomes in the absence of Fz/DFz2 activities, wing discs were labeled with the endosomal marker Texas-red dextran (Entchev et al., 2000). As shown in Figs. 5A–C, more than 50% of Wg puncta co-localize with red dextran, indicating that Wg is internalized in the absence of Fz/DFz2 activities. These observations are consistent with previous results in the embryo (Muller et al., 1999), and altogether suggest that internalization of Wg can be accomplished by proteins other than Fz/DFz2. Interestingly, this observation contrasts with the role of HSPGs in Wg distribution since wing discs lacking GAGs show alteration in Wg puncta in receiving cells (Figs. 1 and 2).

Next, we examined the extracellular distribution of Wg in *fz DFz2* mutant clones. Interestingly, we detected accumulation of extracellular Wg throughout these clones, thus revealing that Wg can bind to the cell surface and that Fz/DFz2 receptors are required somehow for Wg degradation (Figs. 5D–F). To exclude the possibility that accumulation of extracellular Wg resulted from increased *wg* expression or secretion in *fz DFz2* clones that cross D/V boundary, small clones that do not include the D/V boundary were generated. We detected accumulation of extracellular Wg in these clones (Figs. 5G–I), which is reminiscent of the finding that overexpression of a dominant-negative form of *DFz2* (*ΔDFz2-GPI*) driven by *en-Gal4* in embryonic tissue prevents Wg decay within the *en* domain (Dubois et al., 2001). Indeed, Dubois et al. (2001) proposed that endocytosis of a Wg/receptor complex is responsible for down-regulating Wg levels. Further, because Wg is still organized in a graded manner in these clones, as shown by the distribution of the Wg puncta (Figs. 5A–C), it indicates that Wg movement can occur in the absence of Fz/DFz2.

We note that there is a third member of the Frizzled family encoded by *DFz3* that could influence the distribution of Wg in tissues. *DFz3* expression is similar to that of *wg*, and a constitutively activated form of Arm up-regulates its expression in the wing disc, suggesting that *DFz3* is transcriptionally regulated by Wg signaling (Sato et al., 1999; Sivasankaran et al., 2000). Based on these observations, we would expect little or no DFz3 protein to be

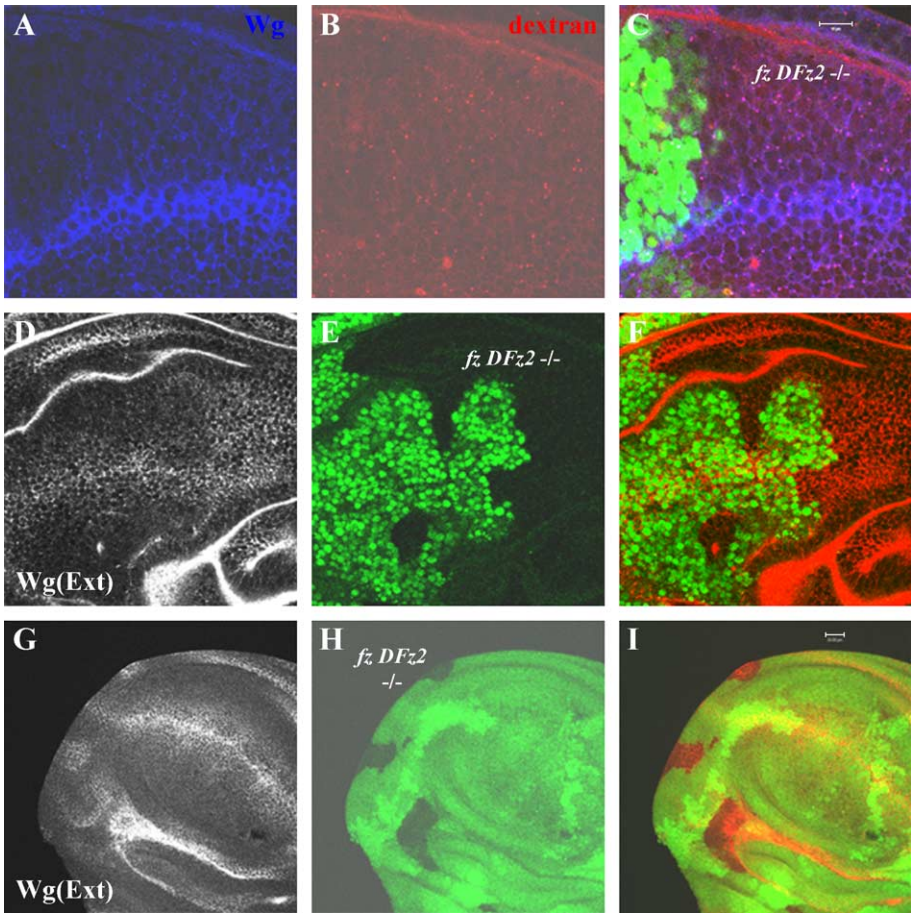

Fig. 5. Fz receptors and Wg movement. (A–F) Distribution of intracellular or extracellular Wg proteins (purple, gray) in *fz DFz2* double-mutant clones. Mutant clones are marked by the absence of GFP. (A–C) In *fz DFz2* double-mutant clones, an expansion of *wg* expression is observed, which is thought to result from the *wg* "self-refinement" process (Rulifson et al., 1996). In addition, the distribution of Wg puncta is not affected, suggesting that the activity of Fz receptors is not required for Wg spreading. Note that Wg puncta in *fz DFz2* mutant cells co-localize with Texas-red dextran (red), indicating that Wg is actually internalized in the mutant clone. (D–I) Wg expression is visualized in *fz DFz2* mutant clone by the extracellular (Ext) labeling method. Accumulation of extracellular Wg in *fz DFz2* mutant clones is observed, indicating Wg can bind to another cell surface receptor(s), and that Fz proteins are required for Wg degradation. The bright lines detected in D and F are due to folds in the discs and are not due to Wg staining. Scale bars: 10 μm (in C for A–C); 20 μm (in I for G–I).

present in cells that lack Fz/DFz2 activity, suggesting that internalization of Wg in *Fz/DFz2* mutant cells is unlikely to be mediated by DFz3. Another candidate that could affect Wg distribution is Arrow, which is a *Drosophila* homolog of a low-density lipoprotein (LDL)-receptor-related protein (LRP) and has been shown to be essential in Wg-receiving cells receiving (Wehrli et al., 2000). However, because a soluble form of the Arrow fails to bind Wg and Fz receptors in vitro (Wu and Nusse, 2002), and that Arrow functions after DFz2 engages Wg (Wehrli et al., 2000), it is unlikely that Wg internalization in *Fz/DFz2* mutant cells is mediated by Arrow. Finally, as is case for FGF endocytosis (Gleizes et al., 1995), HSPGs themselves possibly play a role in Wg internalization.

In summary, we found that there is a Fz/DFz2 receptor-independent mechanism that organizes Wg distribution, and that Fz/DFz2 proteins play a role in Wg gradient formation by decreasing the level of extracellular Wg. Regulation of extracellular Wg levels by Fz/DFz2 may occur through receptor-mediated endocytosis (Dubois et al., 2001), or by some other mechanisms. If Wg degradation occurs by receptor-mediated endocytosis, it indicates that there may exist more than one way to generate Wg puncta as these are still present in the absence of Fz/DFz2 receptor activity. Our findings also emphasize that the amount of Fz/DFz2 receptors at the cell surface must be precisely regulated to achieve the proper spreading of Wg, an observation that is underscored by the transcriptional down-regulation of *DFz2* expression near the source of Wg (Cadigan et al., 1998).

DFz2 and Wg distribution

To further examine the role of Fz/DFz2 receptors in Wg gradient formation, we overexpressed *DFz2* at the D/V boundary using the *C96-Gal4* driver (see Gusfafson and Boulianne, 1996), and examined the effect on Wg distribution and wing patterning. Here, we decided to focus our analysis on DFz2 as DFz2 has been previously shown to bind Wg with high affinity and to stabilize it (Cadigan et al., 1998). Further, *DFz2* expression is down-

regulated by Wg signaling, and this regulation has been shown to play a critical role in the overall shape of the Wg gradient (Cadigan et al., 1998). Interestingly, ectopic expression of *DFz2* resulted in wing notching and ectopic bristles at the wing margin of adult wing (Fig. 6A). Previous studies have shown that wing nick phenotypes resulted from an inhibition in Wg signaling activity (Couso et al., 1994) while the presence of ectopic bristles on the wing blade corresponds to an increase in Wg signaling (Axelrod et al., 1996; Zhang and Carthew, 1998). Thus, based on the wing phenotypes, it appears that overexpression of *DFz2* paradoxically both increases and decreases Wg signaling.

Overexpression of the *DFz2* could interfere with Wg signaling and its distribution in a number of ways. For example, an increase in DFz2 could increase the efficiency of Wg signaling, if the amount of receptor is limiting. Further, as *wg* expression in the wing disc is restricted to the D/V margin, and Wg diffuses from it, trapping of Wg near these cells most likely will have an effect on Wg short- and long-range activity as the shape of the Wg gradient will be disrupted. To distinguish between these possibilities, we examined Wg distribution in discs with clones of cells that overexpress *DFz2* at the D/V boundary. These clones were associated with two effects on Wg distribution. First, the level of Wg was increased in the clones of cells where *DFz2* was overexpressed (Figs. 6B,C), indicating that an increase in the level of DFz2 in receiving cells leads to an increase in trapping extracellular Wg. This observation is consistent with the occurrence of extra bristles on the wing blade since they reflect high levels of Wg signaling activity. Second, we detected a dramatic reduction in Wg puncta in WT cells

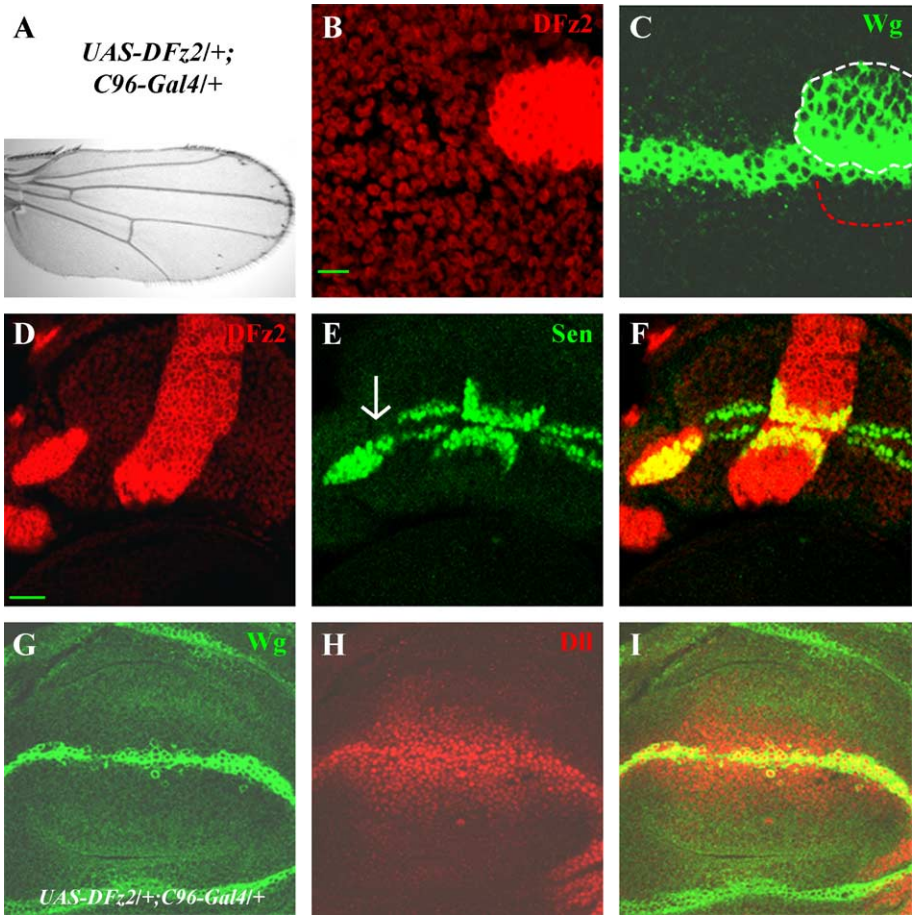

Fig. 6. Function of DFz2 in Wg distribution. (A) Ectopic expression of *DFz2* using the *C96-Gal4* line, which drives Gal4 expression at the D/V boundary, results in a wing notching phenotype and formation of extra bristles close to the wing margin, demonstrating that overexpression of DFz2 paradoxically increases and decreases Wg signaling activity. (B, C) Cells overexpressing *DFz2* are detected using the anti-DFz2 antibody (red, B) and delineated by the white dotted lines (C). Cells overexpressing *DFz2* near the D/V boundary have two effects on Wg distribution. First, Wg (green) accumulation is observed in cells overexpressing *DFz2*, indicating that an increase in the level of DFz2 leads to an increase in trapping of extracellular Wg. Second, less Wg puncta are detected in WT cells (in the region marked by the red dotted line) located next to the clone of cells overexpressing *DFz2*, indicating that Wg movement is impaired as a result of the excess trapping of Wg by cells overexpressing *DFz2*. (D–F) Cells overexpressing *DFz2* are detected using the anti-DFz2 antibody (red), and the sensory organ precursor cells were detected by anti-Sen antibody (green). Complete loss of *sen* expression is observed in WT cells located adjacent to the cells overexpressing *DFz2* (arrow in E). (G–I) Ectopic expression of *DFz2* using the *C96-Gal4* driver causes a decrease in *Dll* expression (red). Note the reduced Wg puncta (green) in receiving cells (G). Scale bars: 10 μm (in B for B, C); 20 μm (in D for D–F).

located adjacent to the cells overexpressing *DFz2* (Figs. 6B,C), suggesting that Wg movement from the D/V margin into the wing blade is impaired as a result of the excess trapping of Wg by cells that overexpress *DFz2*. To demonstrate that Wg accumulation correlates with an increase in Wg signaling and that the absence of Wg puncta correlate with an absence of Wg signaling, we examined the effect of *DFz2* overexpression on the expression of the *sen* (Figs. 6D–F). We found that *sen* expression is expanded in cells overexpressing DFz2, yet *sen* is not expressed in WT cells near a clone of cells overexpressing *DFz2* (Fig. 6E, arrow). This is consistent with the observation that more Wg can be detected in cells overexpressing *DFz2* and that less Wg puncta are present in WT cells near a clone of cells overexpressing *DFz2* (Figs. 6B,C).

We, as well as others, have proposed that Fz proteins contribute to Wg turnover (Dubois et al., 2001). Thus, it is intriguing to note that overexpression of DFz2 leads to an accumulation of extracellular Wg. This may reflect saturation of the endocytotic pathway when DFz2 is overexpressed or an inability of the regulatory pathways that normally control Dfz2 endocytosis in the wing disc to appropriately respond under this overexpressed condition. Another possibility is HSPGs themselves might play role important role Wg endocytosis and the stoichiometry of Fz to HSPGs is essential to promote proper Wg internalization. Detailed biochemical and cell biological studies are now required to clarify the role(s) of these receptors in Wg movement.

Finally, we examined whether overexpression of *DFz2* at the D/V boundary could affect long-range activity of Wg, using wing disc overexpressing *DFz2* driven by *C96-Gal4* driver. Interestingly, *Dll* expression is dramatically shortened in wing disc overexpressing *DFz2* at the D/V boundary (Figs. 6G–I) when compared to that of WT disc (Fig. 3B). We conclude that DFz2 has multiple roles in Wg signaling: First, it transduces Wg signaling and its level is limiting in amount; and second, it affects Wg short- and long-range activity by modulating the availability of extracellular ligand.

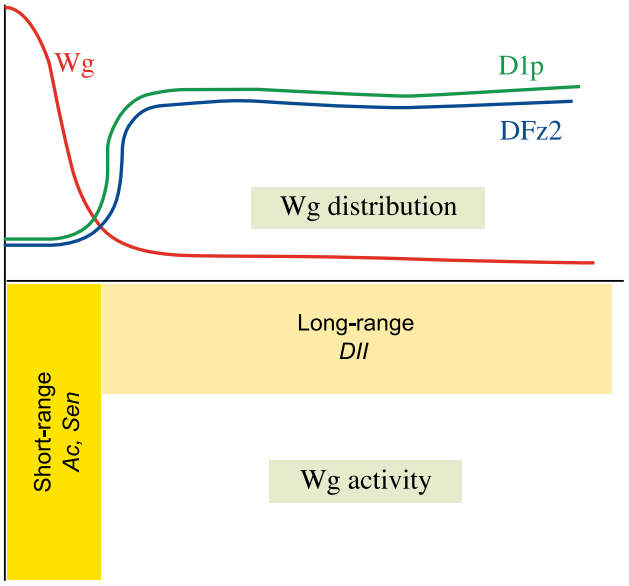

Fig. 7. Schematic diagram showing relationship between Wg activity and Dlp distribution in the wing imaginal disc. Short-range Wg signaling induces the expression of the zinc finger transcription factor *senseless* (*sen*) in the sensory organ precursors (SOPs) along the presumptive wing margin. Long-range Wg signaling controls the expression of *Distal-less* (*Dll*) within the wing blade. A significant decreased level of Dlp is observed at the D/V border. This domain of low-Dlp expression correlates with the region where high level of Wg signaling is required to induce the expression of short-range target genes, suggesting that Dlp negatively influence Wg activity where Wg level is high.

Working model

In this study, we have analyzed the respective roles of HSPGs and Fz/DFz2 receptors in Wg distribution and gradient formation. Interestingly, we found that loss of Dlp activity significantly increased the level of Wg activity in S2R+ cells upon Wg induction, indicating that Dlp acts as a negative regulator in Wg signaling and that it is not required for transducing the Wg signal. Interestingly, our in vivo results show that Dlp protein levels are low near the D/V boundary. Thus, low levels of Dlp near the source of Wg production may allow for activation of high threshold Wg target gene (Fig. 7). It is of interest to note that Notum is highly expressed along the D/V boundary (Giralez et al., 2002), which would be predicted to further diminish HSPGs activity. In addition, we found that Dlp positively influences Wg signaling in S2R+ cells when Wg is not induced, suggesting that it is required for Wg signaling in cells where Wg level is low. A possible explanation for this result is that Dlp may act as a co-receptor that traps/stabilizes extracellular Wg and facilitates its association with signal transducing Fz receptors. In the wing imaginal disc, given that Dlp is required for Wg signaling in cells where Wg levels are low, HSPG activity is possibly required for Wg signaling by somehow facilitating Wg movement. The binding of extracellular Wg to the low-affinity HSPG receptors in receiving cells may result in the association of Wg to cell membranes. Ligand movement could then occur by a mechanism that directly involves HPSGs where subsequent cycles of Wg dissociation/reassociation with HSPGs might promote the movement or required other yet to be identified extracellular molecules. To distinguish these possibilities, the role of HSPGs in Wg movement will require further detailed analysis. Regardless, our results clearly indicate that the primary role of HSPGs is to sequester and/or stabilize extracellular Wg in receiving cells. The imposition of the HSPG-mediated Wg accumulation and the Fz-dependent degradation mechanism would thus contribute to the Wg morphogen gradient. It is important to note that the expression levels of some of the critical components of each systems (i.e., *dally*, *DFz2*) are also regulated by the Wg pathway itself, indicating that the slope of the Wg

gradient is established by the delicate balance between these two systems.

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