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The Core Protein of Glypican Dally-Like Determines Its Biphasic Activity in Wingless Morphogen Signaling

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SUMMARY

Dally-like (Dlp) is a glypican-type heparan sulfate proteoglycan (HSPG), containing a protein core and attached glycosaminoglycan (GAG) chains. In *Drosophila* wing discs, Dlp represses short-range Wingless (Wg) signaling, but activates long-range Wg signaling. Here, we show that Dlp core protein has similar biphasic activity as wild-type Dlp. Dlp core protein can interact with Wg; the GAG chains enhance this interaction. Importantly, we find that Dlp exhibits a biphasic response, regardless of whether its glycosylphosphatidylinositol linkage to the membrane can be cleaved. Rather, the transition from signaling activator to repressor is determined by the relative expression levels of Dlp and the Wg receptor, Frizzled (Fz) 2. Based on these data, we propose that the principal function of Dlp is to retain Wg on the cell surface. As such, it can either compete with the receptor or provide ligands to the receptor, depending on the ratios of Wg, Fz2, and Dlp.

INTRODUCTION

The morphogen model is a well-established mechanism to explain the formation of complex cell and tissue patterns during development (Ashe and Briscoe, 2006; Lawrence and Struhl, 1996). Morphogens are produced from a localized source, and form concentration gradients that provide positional information for cell fate specifications. In the last two decades, it has been firmly established that a small number of secreted signaling molecules, including members of the Wingless (Wg)/Wnt, Hedgehog (Hh), and bone morphogenetic protein (BMP) families, act as morphogens (Tabata and Takei, 2004). The mechanisms of their gradient formation and interpretation are of fundamental interest, but are highly complex and not well understood (Lander, 2007). Recently, increasing numbers of cell surface and extracellular cofactors have been shown to bind morphogens and to regulate their distribution and signaling. In *Drosophila*, Dally, Dally-like (Dlp), and Lipoprotein are involved in Wg signaling (Mikels and Nusse, 2006); Dally, Dlp, Interference hedgehog (Ihog), Shifted (Shf) and Lipoprotein are involved in Hh signaling (Jiang and Hui, 2008); Dally, Dlp, Short gastrulation (Sog), and Crossveinless (CV)-2 are involved in BMP signaling (Bier, 2008). In vertebrates, there are even more extracellular components involved. Now, it becomes increasingly important to understand how these cofactors fine-tune morphogen signaling strength, range, and robustness during development.

In this article, we focus on the mechanisms underlying the regulation of Wg morphogen signaling by Dlp. Dlp is a glypican member of the heparan sulfate proteoglycans (HSPGs), which are present on the cell surface and in the extracellular matrix (Bernfield et al., 1999; Lin, 2004). HSPGs are composed of a protein core to which heparan sulfate (HS) glycosaminoglycan (GAG) chains are attached. HS GAG chains are linear polysaccharide chains expressing a multitude of sulfation patterns. They can bind a wide variety of extracellular ligands, including Wg (Bernfield et al., 1999; Reichsman et al., 1996). Genetic analyses have found that Wg signaling is defective in mutant encoding HS GAG biosynthesis enzymes, such as *sugarless* (*sgl*) and *sulfateless* (*sfl*) (Hacker et al., 2005; Lin, 2004). Wg protein level is reduced in the HSPG-deficient cells, suggesting that the movement or stability of Wg morphogen depends on HS GAG chains (Baeg et al., 2001; Bornemann et al., 2004; Han et al., 2004a; Takei et al., 2004). Further genetic studies have demonstrated that two glypicans, Dally and Dlp, play cooperative and distinct roles in modulating Wg gradient and signaling. Removal of both Dally and Dlp leads to strong reduction of extracellular Wg, suggesting that Dally and Dlp are the major core proteins providing effective GAG chains for Wg signaling (Han et al., 2005). However, various studies suggest that Dally and Dlp perform distinct activities in Wg signaling. The *dally* mutants exhibit wing margin defects, and show genetic interactions with Wg signaling components, arguing that Dally plays a positive role in Wg signaling (Franch-Marro et al., 2005; Fujise et al., 2001; Han et al., 2005; Lin and Perrimon, 1999). Both Dally and Dlp bind Wg in cell culture; however, only Dlp overexpression causes Wg accumulation in the wing discs (Baeg et al., 2001; Franch-Marro et al., 2005; Han et al., 2005). These observations are consistent with a classical coreceptor role for Dally in Wg signaling.

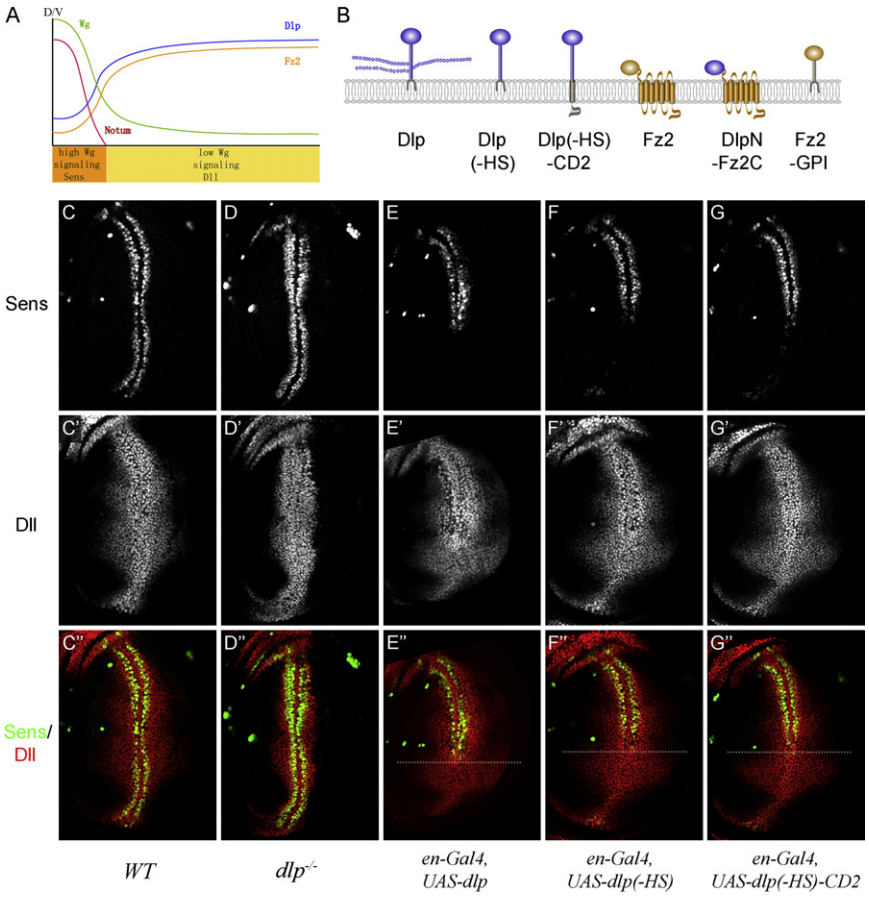

Figure 1. Dally-Like Core Protein Has Biphasic Activity in Wingless Signaling

(A) Schematic diagram of Wingless (Wg) protein distribution, *fz2*, *dlp*, and *notum* expression patterns in wing disc.

(B) Major Dally-like (Dlp) and Frizzled (Fz) 2 constructs used in this study.

(C–C″) The expression of *sens* (C) and *dll* (C′) were analyzed by antibody staining in wild-type wing discs.

(D–D″) The expression of *sens* (D) and *dll* (D′) in *dlp* homozygous mutant discs. The domain of *sens* expression is broadened, and the domain of *dll* expression is significantly narrowed. Also see Figure S1 for quantifications. Wing imaginal discs in all the figures are oriented anterior to the top and dorsal to the left, except in Figures 2 and 3.

(E–G″) Expression of Dlp (E–E″), Dlp(−HS) (F–F″), or Dlp(−HS)-CD2 (G–G″) in the posterior compartment (below the dashed line) by *en-Gal4* diminishes *sens* expression (E, F, and G) and expands *dll* expression domain (E′, F′, and G′).

Dally could present Wg to Frizzled (Fz) 2 signaling receptor, leading to activation of signaling and rapid degradation of the complex (Franch-Marro et al., 2005; Lin and Perrimon, 1999).

Dlp has a more intriguing activity in regulating Wg signaling and gradient. In the wing disc, expression of both Dlp and Fz2 are repressed by Wg signaling, thus forming an inverse pattern to that of Wg (see Figure 1A for diagram of Wg, *fz2*, *dlp*, and *notum* expression patterns) (Cadigan et al., 1998; Han et al., 2005). Both loss-of-function and gain-of-function studies suggest that Dlp acts as a positive regulator in the regions of the wing disc distant from the site of Wg production (low Wg and high Fz2 levels), while it also acts as a negative regulator near the site of Wg production (high Wg and low Fz2 levels) (Baeg et al., 2004; Franch-Marro et al., 2005; Han et al., 2005; Hufnagel et al., 2006; Kirkpatrick et al., 2004; Kreuger et al., 2004). How do we understand this biphasic activity of Dlp in Wg signaling? One current model proposes that the biphasic activity of Dlp is controlled by *notum* (also known as *wingful*), which encodes a member of the α/β-hydrolase superfamily (Gerlitz and Basler, 2002; Giraldez et al., 2002). Notum acts as a Wg antagonist, and is induced by high-level Wg signaling in the dorsal/ventral (D/V) boundary (Figure 1A). Biochemical experiments show that Notum can induce cleavage of Dlp protein at the level of its glycosylphosphatidylinositol (GPI) anchor, which leads to shedding of Dlp from the cell surface. Thus, Notum-mediated cleavage might convert Dlp from a membrane-tethered coreceptor to a secreted antagonist in areas close to the D/V boundary (Kreuger et al., 2004). On the other hand, another model suggests that Dlp captures Wg, but instead of presenting it to Wg signaling receptors expressed in the same cell, it passes Wg to neighboring cells (Franch-Marro et al., 2005). In this way, Dlp can inhibit Wg signaling locally by competing with Wg receptors, but enhance Wg signaling by promoting Wg gradient formation to the distal part of the disc (Hufnagel et al., 2006). Further genetic and biochemical experiments are required to define the mechanisms underlying Dlp's biphasic activity.

Here, we present evidence that Dlp's core protein contributes the main activity of Dlp in Wg signaling. Dlp core protein can bind Wg and show biphasic activity in Wg signaling. Importantly, we demonstrate that Dlp can get a biphasic response without Notum cleavage, and that the ratio of Dlp and Fz2 can determine the biphasic activity of Dlp in Wg signaling. On the basis of our data, we proposed a model, referred to as an exchange factor model, in which Dlp's major function is to retain Wg on the cell surface; it might either compete with receptor, or provide ligands for the receptor, depending on its levels.

RESULTS

Biphasic Activities of Dlp and Its Core Protein in Wg Signaling

In the wing disc, Wg is secreted from the D/V border, and induces the expression of Wg-target genes in a concentration-dependent manner. Wg induces *sens* expression at short range, while it activates *dll* at long range (Figures 1A and 1C–1C″) (Neumann and Cohen, 1997; Nolo et al., 2000; Zecca et al., 1996). In *dlp* homozygous mutant discs, the domain of *sens* expression is broadened, while the range of *dll* expression is significantly narrowed (Figures 1D–1D″; see quantifications in Figures S1A and S1B available online). In contrast, overexpression of *UAS-dlp* in

the posterior compartment of the disc by *en-Gal4* eliminates *sens* expression, while it expands the *dll* expression range (Figures 1E–1E″; see quantifications in Figures S1C and S1D). Although the *dll* expression range is enhanced, the *dll* expression level is reduced in areas close to the D/V boundary (Figure 1E′). These results suggest that Dlp acts as a positive cofactor to enhance Wg signaling activity in areas distant from the Wg source, while it acts as a negative cofactor to suppress Wg signaling in areas close to the Wg source (Franch-Marro et al., 2005; Hufnagel et al., 2006; Kirkpatrick et al., 2004; Kreuger et al., 2004).

The biphasic activity of Dlp is apparently different from that of Dally, which only acts as a positive cofactor for Wg signaling (Franch-Marro et al., 2005; Han et al., 2005). To examine the mechanism underlying the biphasic activity of Dlp, we first attempted to determine the protein domain(s) required. Dlp is composed of three functional domains, including a protein core, attached HS GAG chains, and a GPI anchor (Baeg et al., 2001). We constructed a Dlp core protein expression vector, *UAS-dlp(−HS)*, lacking all of the GAG attachment sites (see Figure 1B for sketches of major constructs used in this study). To evaluate whether Dlp(−HS) is indeed devoid of HS chains, we expressed Dlp(−HS) in vivo and stained with 3G10 antibody, which recognizes an HS epitope produced by enzymatic digestion with heparitinase (David et al., 1992; Kirkpatrick et al., 2006). While expression of wild-type Dlp in the posterior compartment strongly enhances 3G10 staining, expression of Dlp(−HS) does not increase the staining, suggesting that Dlp(−HS) is indeed lacking HS modifications (Figure S2). Next, we tested the in vivo activity of Dlp(−HS). Interestingly, expression of Dlp(−HS) in the posterior compartment has a similar biphasic response to that of wild-type Dlp, although the repression activity of Dlp(−HS) is somewhat weaker (Figures 1F–1F″). This result suggests that the activity of Dlp in Wg signaling is largely due to its core protein. We further examined the role of the GPI anchor of Dlp in Wg signaling. We constructed Dlp(−HS)-CD2, in which the Dlp's GPI anchor is replaced by a transmembrane protein, rat CD2 (Strigini and Cohen, 1997). Expression of Dlp(−HS)-CD2 shows very similar biphasic activity to that of Dlp(−HS) (Figures 1G–1G″), arguing that the GPI anchor of Dlp is not essential for its activity in Wg signaling. Our results are different from those of a recent work that suggested a role of the GPI anchor of Dlp in long-range Wg signaling (Gallet et al., 2008).

Dlp Core Protein Interacts with Wg

Next, we examined whether the Dlp core protein can bind Wg. First, we incubated Wg-conditioned medium with *Drosophila* S2 cells transfected with *dlp-GFP*, *dlp(−HS)-GFP*, *dlp(−HS)-CD2-GFP*, and *GFP-GPI* control. Wg can bind to cells transfected with the *dlp* constructs, but not those transfected with *GFP-GPI* (Figures 2A–2D′). However, *dlp-GFP* cells accumulate more Wg on the cell surface than *dlp(−HS)-GFP* or *dlp(−HS)-CD2-GFP* cells, suggesting that Dlp has a greater ability to bind Wg than the Dlp core protein. Second, we performed coimmunoprecipitation (co-IP) experiments in S2 cells expressing *dlp*, *dlp(−HS)*, or *dlp(−HS)-CD2* with *wg-GFP*. Consistent with the cell-binding assay, Wg can be coprecipitated with Dlp, Dlp(−HS), or Dlp(−HS)-CD2, but more Wg is coprecipitated by Dlp than by the other two proteins (Figure 2H, arrow). On the other hand, Wg does not coprecipitate with Connectin, a GPI-linked protein that has not been implicated in Wg signaling (Nose et al., 1992) (Figure 2J).

We further tested whether the Dlp core protein can bind Wg in vivo. Ectopic expression of Dlp, Dlp(−HS) or Dlp(−HS)-CD2 can cause Wg accumulation on the cell surface (Figures 2L–2N). Together with the in vitro assay, these experiments argue that the GPI anchor of Dlp is dispensable for Wg binding. In addition, expression of GFP-GPI protein or Syndecan (another HSPG protein) does not cause Wg accumulation in the wing discs (Figures 2R and 2S), suggesting Dlp specifically interacts with Wg. We further determined the Dlp domain required for Wg binding. DlpΔGAG, which lacks the GAG attachment domain, still interacts with Wg in all the assays (Figures 2E, 2H, and 2O). However, DlpΔN, devoid of the N-terminal domain, fails to bind Wg (Figures 2G, 2I, and 2Q), suggesting that the N-terminal domain of Dlp is required for this interaction. This failure to interact with Wg is not due to instability of DlpΔN on the cell membrane or different subcellular localization of this protein (Figure S3). Indeed, when the N-terminal domain of Dlp is linked to CD2, this protein (DlpN-CD2) still retains the ability to interact with Wg in various assays (Figures 2F, 2H, and 2P). Finally, we tested the signaling activities of these proteins in the wing discs. Consistent with their abilities to interact with Wg, DlpΔGAG and DlpN-CD2 have biphasic activities, while DlpΔN has no activity in Wg signaling (Figures 2T–2V′).

Collectively, these results suggest that the core protein of Dlp can interact with Wg, while the attached HS chains can enhance the Wg-binding capability of Dlp. The GPI anchor of Dlp is not important for this interaction, while the N-terminal domain of Dlp is essential for its interaction with Wg.

Colocalization of Dlp and Wg in Endocytic Vesicles Is Irrelevant to Dlp's Activity

We further examined the subcellular localizations of different forms of Dlp. For this purpose, we generated GFP-tagged versions of Dlp, Dlp(−HS), and Dlp(−HS)-CD2, in which the GFP tag is inserted into the same position of Dlp proteins as described previously (Baeg et al., 2004) (see Experimental Procedures for details). These proteins have similar activities as nontagged forms (Figure S4). We then expressed Dlp-GFP, Dlp(−HS)-GFP, and Dlp(−HS)-CD2-GFP in discs by *en-Gal4*. In Dlp-GFP-expressing cells, Wg accumulates mainly on the cell membrane, while, in Dlp(−HS)-GFP- and Dlp(−HS)-CD2-GFP-expressing discs, it is less accumulated on the cell membrane, but more in punctate vesicles (Figures 3A, 3H, and 3O), which colocalize with the endocytic marker Texas red dextran (Rives et al., 2006) (Figure 3D, 3K, and 3R). Previous studies have suggested that Wg internalization is mediated through its interaction with the Fz2 receptor (Piddini et al., 2005). Thus, our data are consistent with the view that Dlp retains Wg on the membrane and competes with Fz2 for Wg binding. Because the wild-type Dlp has stronger binding affinity for Wg than the core protein of Dlp, more Wg protein is retained on the surface of Dlp-expressing cells, thereby causing reduced levels of internalized Wg vesicles. It is worthwhile to note that Dlp-GFP and Dlp(−HS)-GFP are present in many endocytic vesicles, while Dlp(−HS)-CD2-GFP virtually does not exist in vesicular structures (Figures 3B, 3E, 3I, 3L, 3P, and 3S). As a result, only a small portion of Dlp-GFP colocalizes with Wg in endocytic vesicles (Figures 3F and 3G);

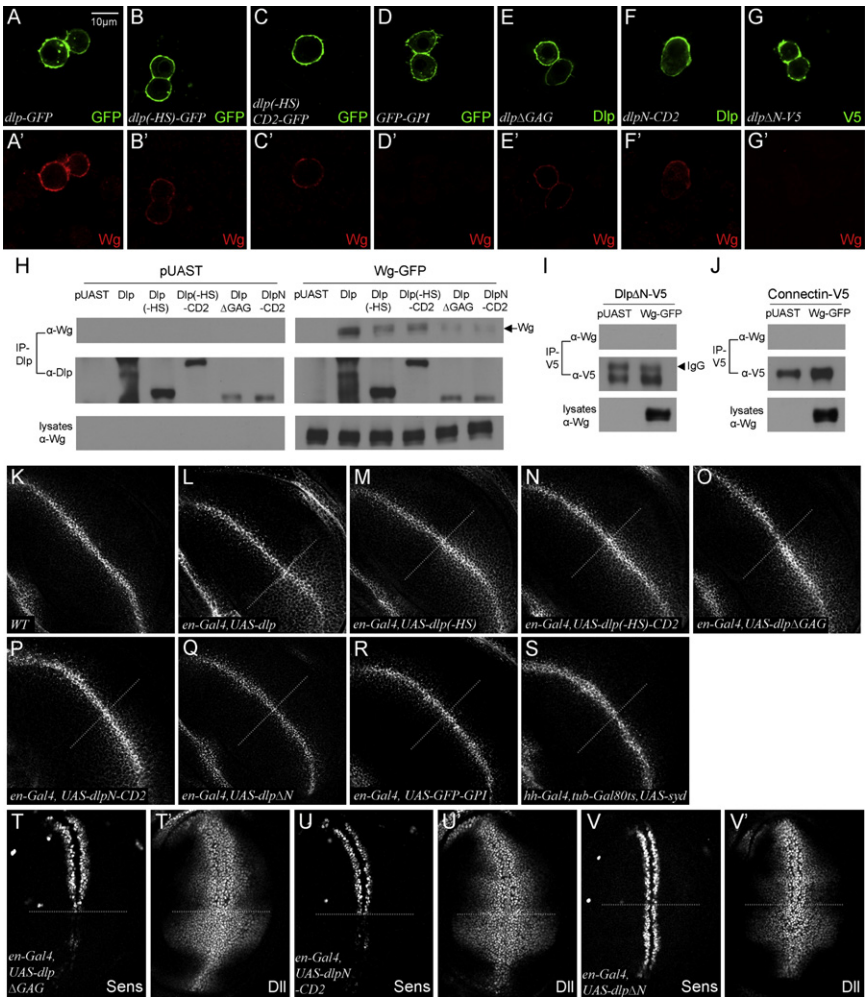

Figure 2. Dlp Core Protein Can Interact with Wg In Vitro and In Vivo

(A–C′) Transfection of *dlp-GFP*, *dlp(−HS)-GFP*, or *dlp(−HS)-GFP-CD2* in S2 cells causes accumulation of exogenous Wg at the cell surface. Notice that *dlp-GFP* expression cells accumulate more Wg than *dlp(−HS)-GFP* and *dlp(−HS)-GFP-CD2* expression cells (A′, B′, and C′).

(D′) The control cells transfected with *GFP-GPI* plasmid do not cause Wg accumulation at the cell surface.

(E–G′) Cells transfected with *dlp-ΔGAG* or *dlpN-CD2* can bind exogenous Wg (E′ and F′), but cells transfected with *dlpΔN-V5* do not cause Wg binding (G′). Transfected cells are recognized with α-GFP (A–D), α-Dlp (E and F), or α-V5 (G), respectively. Scale bar, 10 μm.

(H–J) Wg can coimmunoprecipitate (co-IP) with Dlp core protein. Top and middle panels: S2 cells were transfected with indicated expression vectors, and cell lysates were IP and analyzed by Western blotting with the antibodies indicated. Bottom panel: the amount of Wg-GFP in 5% of cell lysates input was assessed by Western blot. Dlp forms a smear typical of heparan sulfate proteoglycans, while Dlp(−HS) displays a sharp band in protein gel, indicating it is a nonglycanated form. Note that more Wg-GFP is coprecipitated with Dlp than Dlp(−HS) (arrow).

(K–S) Dlp core protein causes Wg accumulation in vivo. Various transgenes are expressed in the posterior compartment of the wing discs (on the right of the dashed line) and analyzed for their effects on Wg distribution. Compared to wild-type disc (K), expression of Dlp (L), Dlp(−HS) (M), Dlp(−HS)-CD2 (N), Dlp-ΔGAG (O), or DlpN-CD2 (P) causes Wg accumulation in the posterior compartment. Expression of Dlp-ΔN (Q), GFP-glycosylphosphatidylinositol (GPI) (R), or Syndecan (S) does not affect Wg distribution. All transgenes are driven by *en-Gal4*, except *syndecan* is induced by *hh-Gal4-Gal80ts* for 24 hr at 30°C, because induction of *syndecan* by *en-Gal4* leads to early lethality. The wing discs are oriented dorsal bottom left, anterior top left.

(T–V′) Various isoforms of *dlp* transgenes are induced in the posterior compartment of the wing discs (below the dashed line) and analyzed for their effects on *sens* and *dll* expression. While DlpΔGAG (T–T′) and DlpN-CD2 (U–U′) remain the biphasic activities to repress *sens* and expand *dll*, DlpΔN has no effect on *sens* or *dll* expression (V–V′).

much Dlp(−HS)-GFP colocalizes with Wg in vesicles (Figures 3M and 3N), but almost no Dlp(−HS)-CD2-GFP colocalizes with Wg in vesicles (Figures 3T and 3U). Together, our results argue that, although the GPI anchor of Dlp affects the sorting of Dlp proteins into endocytic compartments, it is not important for its activity in Wg signaling.

Our result differs from that in a recent publication arguing that the GPI anchor of Dlp is essential for Wg transcytosis and long-range signaling (Gallet et al., 2008). In that study, the authors generated a GFP-Dlp-CD2 construct, and found its activity significantly different from their GFP-Dlp. Surprisingly, we found their GFP-Dlp-CD2 construct has very similar activity to the Dlp-GFP construct in our experiments. As shown in Figure 4, expression of GFP-Dlp-CD2 by *en-Gal4* results in reduction of *sens*, but expansion of *dll* expression (Figures 4A–4A‴). Similarly, expression of GFP-Dlp-CD2 by *ap-Gal4* does not reduce *dll* expression (Figures 4B–4B‴). Furthermore, GFP-Dlp-CD2 does not induce a more severe wing defect than our Dlp-GFP (Figures 4D–4G). Similar to our Dlp(−HS)-CD2-GFP, their GFP-Dlp-CD2 also does not form vesicles, and thus does not colocalize with Wg vesicles (Figures 4C–4C″). In conclusion, our data suggest that Dlp's role in Wg signaling mainly depends on its activity on the cell membrane; its colocalization with Wg in endocytic vesicles is irrelevant to Dlp's activity in Wg signaling.

DlpN-Fz2C Fusion Protein Acts as a Weak Version of Fz2

So far, we have shown that the Dlp core protein can interact with Wg. If the function of Dlp's core protein is to capture Wg, replacement of the cysteine-rich domain (CRD) in Fz2 by the core protein of Dlp would convert Dlp to a signaling receptor. We tested this hypothesis by making a DlpN-Fz2C fusion protein and expressing it in the wing discs. Expression of Dlp, Dlp(−HS), or Dlp(−HS)-CD2 by *dpp-Gal4* leads to reduction of *sens* in the *dpp* expression domain (Figures 5E–5G). In contrast, induction of Fz2 by *dpp-Gal4* activates *sens* expression

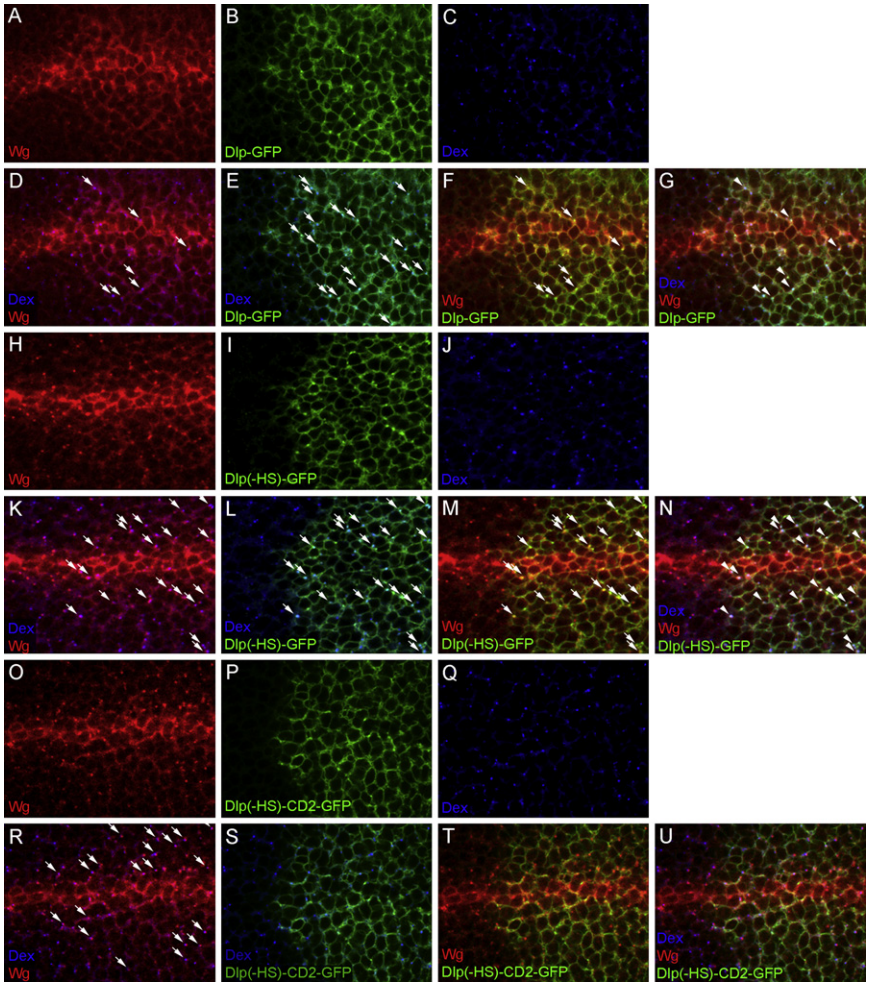

Figure 3. Subcellular Localization of Wg, Dlp/GFP Fusion Proteins, and Endosome Markers

Wg staining (red) and Texas red dextran labeling (blue) in discs expressing Dlp-GFP (A–G), Dlp(−HS)-GFP (H–N), or Dlp(−HS)-CD2-GFP (O–U) (green) in the posterior compartment by *en-Gal4*. Dextran labeling was performed by a 10 min pulse and 20 min chase to visualize the endocytic compartments. Dlp-GFP accumulates more Wg on the cell membrane and in Dlp(−HS)-GFP- and Dlp(−HS)-CD2-GFP-expressing discs, Wg is less accumulated on the cell membrane, but more localized in internalized vesicles (A, D, H, K, O, and R; arrows point to double colocalized vesicles). Qualitatively, in Dlp-GFP expression cells, only 2.2 ± 0.6% of total Wg is in vesicles, while in Dlp(−HS)-GFP and Dlp(−HS)-CD2-GFP cells, 5.9 ± 0.6% and 5.6 ± 0.8% of total Wg are in vesicles, respectively (the latter two are statistically significant from the first; $p < 0.01$, $n = 5$.) On the other hand, Dlp-GFP and Dlp(−HS)-GFP form many internalized vesicles, while Dlp(−HS)-GFP-CD2 almost does not exist in vesicular structures (B, E, I, L, P, and S; arrows point to double colocalized vesicles). Only a small fraction of Dlp-GFP colocalizes with Wg in endocytic vesicles, while many Dlp(−HS)-GFP vesicles colocalize with Wg, and almost no Dlp(−HS)-GFP-CD2 colocalizes with Wg in vesicles (F, G, M, N, T, and U; arrows point to double colocalized vesicles and arrowheads point to triple colocalized vesicles). The wing discs are oriented anterior to the left.

three to four cells further than its normal domain, reflecting elevated Wg signaling activity (Figure 5A) (Cadigan et al., 1998). This signaling activity is abolished in a CRD-deleted form of Fz2, Fz2C (Figure 5B). Interestingly, DlpN-Fz2C expression driven by *dpp-Gal4* can activate *sens* expression up to one to two cells (Figure 5C). This result further supports our view that the function of the Dlp core protein is to bind Wg, and that its binding affinity for Wg is less than that of Fz2 CRD domain.

Presence of Fz2 Converts Dlp from an Inhibitor to an Activator

If the activity of Dlp is to retain Wg on the cell surface, can this action explain Dlp's biphasic function? A previous model suggests that expression of Notum, a negative regulator for Wg in the D/V boundary, could convert Dlp from a coreceptor to a secreted antagonist (Kreuger et al., 2004). It was proposed that Dlp normally acts as a positive coreceptor by providing sources of Wg, while Notum can cleave the GPI anchor of Dlp and release it from the cell surface together with its bound Wg (Kreuger et al., 2004). However, we found that DlpΔGPI, a secreted form of Dlp (similar to the Dlp form cleaved by Notum), fails to act as a repressor for Wg, as its expression does not lead to reduction of Sens levels (Figure 5D). Moreover, this model also cannot explain the dual activities of CD2 forms of Dlp (Figures 1G–1G″ and 4A–4A‴; Figures S4C–S4C‴), since Notum does not cleave CD2 forms of Dlp (Kreuger et al., 2004). These data lead us to consider mechanisms other than Notum to explain the biphasic activity of Dlp.

We propose that the primary role of Dlp is to retain Wg on the cell surface, providing Wg source for Fz2, but also competing with Fz2 for Wg binding. If this is the case, altering the ratios of Dlp and Fz2 might change the activity of Dlp in Wg signaling. We first tested this hypothesis in the wing discs. As mentioned, ectopic expression of Dlp, Dlp(−HS), or Dlp(−HS)-CD2 by *dpp-Gal4* leads to reduced *sens* expression (Figures 5E–5G), while expression of Fz2 leads to activation of *sens* three to four cells wide (Figure 5A). Surprisingly, when Dlp, Dlp(−HS), or Dlp(−HS)-CD2 is coexpressed with Fz2, they constantly activate ectopic *sens* expression up to 11–12 cells wide (Figures 5H–5J). These data imply that Fz2 can utilize Wg provided by Dlp, converting Dlp from a Wg inhibitor to an activator.

Fz2:Dlp Ratio Determines Dlp's Biphasic Activity

We further examined our hypothesis in cultured S2 cells. S2 cells were transfected with a fixed amount of Fz2 expression plasmids and variable amounts of Dlp or Dlp(−HS) expression plasmids. The cells were also cotransfected with a Wg reporter

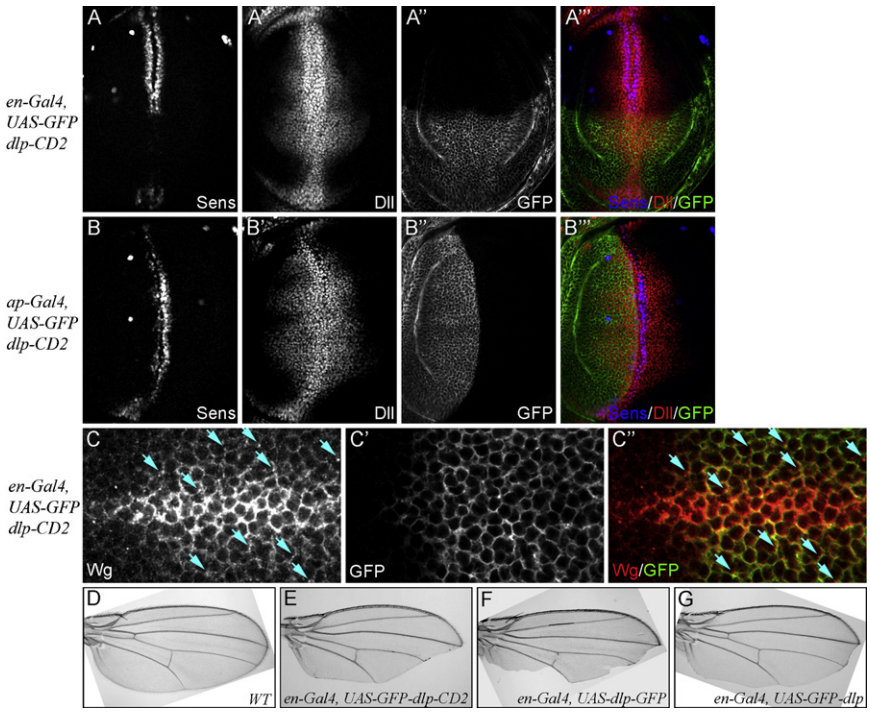

Figure 4. GFP-Dlp-CD2 Has Similar Activity to GPI-Anchored Form of Dlp

(A–A″′) GFP-Dlp-CD2 is expressed in the posterior compartment of the wing disc by *en-Gal4* (A″). It represses *sens* expression (A) and expands the *dll* expression range (A′).

(B–B″′) GFP-Dlp-CD2 is expressed in the D compartment by *ap-Gal4* (B″). It reduces *sens* expression (B), but does not reduce the *dll* expression range (B′).

(C–C″) GFP-Dlp-CD2 is expressed in the posterior compartment by *en-Gal4*, and its subcellular distribution was analyzed together with Wg antibody staining. GFP-Dlp-CD2 forms very few vesicle structures (C′), and thus does not colocalize with Wg vesicles (C and C″, turquoise arrows).

(D–G) GFP-Dlp-CD2 does not induce a more severe wing defect than Dlp-GFP. GFP-Dlp-CD2, Dlp-GFP, and GFP-Dlp are expressed in the posterior compartment by *en-Gal4*. While GFP-Dlp-CD2 expression (E) gives rise to more severe wing defects than GFP-Dlp (G), it does not generate more severe defects than Dlp-GFP (F). Such a discrepancy is because GFP-Dlp has reduced activity due to insertion of GFP tag.

plasmid (12XdTOP), a normalization *Renilla* Luciferase expression plasmid, and then treated with Wg-conditioned medium (DasGupta et al., 2005). As shown in Figures 6A–6B, while a low level of Dlp or Dlp(−HS) promotes Wg signaling, a high level of Dlp or Dlp(−HS) represses Wg signaling activity. This result is consistent with our in vivo data, indicating that Dlp can either compete with Fz2 for available Wg, or provide Wg for Fz2, depending on its levels.

To directly demonstrate the exchange of Wg between Dlp and Fz2, we performed co-IP experiments. S2 cells were transfected with a fixed amount of Wg and Fz2, but variable amounts of Dlp or Dlp(−HS). After the cells were lysed, Fz2 was immunoprecipitated, and the associated Wg was determined by Western blot. As shown in Figures 6C–6D, the total amount of Wg in the lysate increases as the Dlp or Dlp(−HS) amount increases, probably reflecting its ability to stabilize Wg. However, Fz2-bound Wg levels show a biphasic change; while a small amount of Dlp or Dlp(−HS) helps Fz2 gain more Wg, a large amount of Dlp prevents Fz2 from capturing Wg (Figures 6C–6D, arrows). Next, we examined whether Dlp can be pulled down by Fz2 in Co-IP experiments, as a previous study suggests that *Xenopus* Glypican-4 can bind Fz7 in noncanonical Wnt signaling (Ohkawara et al., 2003). However, we did not detect Dlp precipitated by Fz2, suggesting that Dlp does not form a stable complex with Fz2 as a classical coreceptor (Figures 6C–6D).

Dlp's Biphasic Response Changes in Different Wg and Fz2 Concentrations

Collectively, our data suggest that Dlp might either compete with the receptor, or provide Wg ligand for the receptor, depending on its levels. More receptor would bias ligand movement from Dlp to receptor; more Dlp would bias ligand movement toward Dlp and away from the receptor. We refer to such activity of Dlp as "the exchange factor," which was also proposed recently to explain the biphasic BMP signaling activity of CV-2 (Serpe et al., 2008) (Figure 7E).

In the wing disc, Fz2 is expressed in an inverse pattern to that of Wg, with the lowest levels at the D/V boundary (Figure 1A) (Cadigan et al., 1998). Dlp acts negatively in areas close to the D/V boundary, where the Wg level is high and the Fz2 level is low, and positively in areas farther away from the D/V boundary, where the Wg level is low and the Fz2 level is high. To mimic the in vivo situation, we performed the Luciferase experiments for two different Wg or Fz2 levels. Interestingly, as shown in Figure 6E, the biphasic curve switches to the left in the high-Wg situation, which means, for a given amount of Dlp, it is more likely to act as an inhibitor at high Wg concentration, but as an activator at low Wg concentration. In contrast, when we increase the Fz2 amount, the biphasic curve shifts to the right (Figure 6F), suggesting that Dlp is more likely to act as an activator at high Fz2 concentration, but as an inhibitor at low Fz2 concentration. Together, these results are consistent with the in vivo conditions where Dlp acts as an activator when Wg is low and Fz2 is high, but as a repressor in the opposite situation.

Fz2-GPI Has Biphasic Activity in Wg Signaling

To further prove our model, we asked whether any other protein that can exchange with Fz2 for Wg binding has biphasic activity in Wg signaling. One candidate is Fz2-GPI, which contains the Fz2 CRD domain linked to a GPI anchor (Cadigan et al., 1998). It has been shown that expression of Fz2-GPI can reduce Wg-target gene expression in the wing discs by competing with Fz2 for Wg ligand (Cadigan et al., 1998). Indeed, the expression of Fz2-GPI leads to accumulation of Wg on the cell surface, similar to that of Dlp (Figures 7B–7B′). Interestingly,

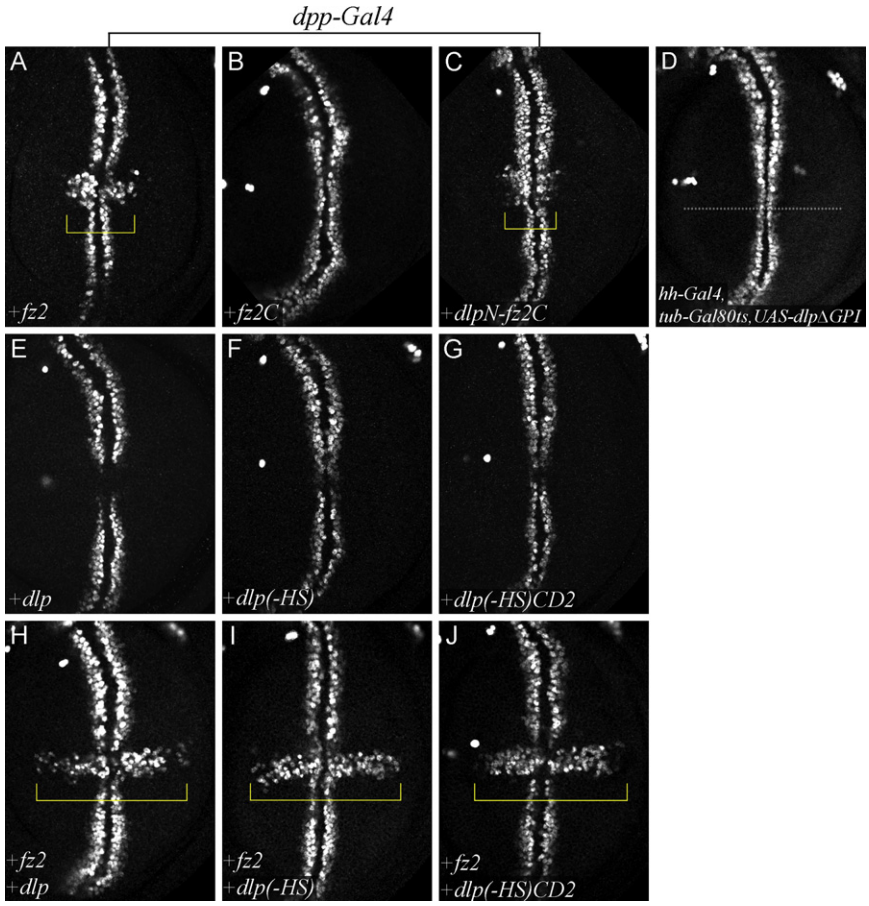

Figure 5. Coexpression of Dlp with Fz2 Converts Dlp from an Inhibitor to an Activator

(A) When Fz2 transgene is expressed in a stripe along the A/P compartment boundary by *dpp-Gal4*, it leads to ectopic *sens* activation three to four cells wide in *dpp* expression domain.

(B) A CRD-deleted form of Fz2, Fz2C, does not induce ectopic expression of *sens* when expressed by *dpp-Gal4*.

(C) Unlike Dlp that represses *sens* expression, the DlpN-Fz2C fusion protein can induce ectopic expression of *sens* up to one to two cells wide.

(D) A GPI-deleted form of Dlp, Dlp-ΔGPI, is expressed in the posterior compartment of the wing disc (below the dashed line), and it does not affect the expression of *sens*. Dlp-ΔGPI is induced by *hh-Gal4*, *tub-Gal80ts* for 24 hr in 30°C, because expression of Dlp-ΔGPI by *en-Gal4* leads to early lethality.

(E–J) Expression of Dlp (E), Dlp(−HS) (F), and Dlp(−HS)-CD2 (G) by *dpp-Gal4* represses *sens* expression in *dpp* expression domain. However, coexpression of Fz2 with Dlp (H), Dlp(−HS) (I), or Dlp(−HS)-CD2 (J) induces ectopic *sens* expression up to 11–12 cells wide.

expression of Fz2-GPI by *hh-Gal4* diminishes *sens* expression, while it expands the *dll* expression domain (Figures 7A–7A″), confirming that Fz2-GPI exhibits biphasic activity in Wg signaling. We further tested its activity in the presence of wild-type Fz2 by *dpp-Gal4*. While expression of Fz2-GPI by *dpp-Gal4* leads to reduction of *sens* (Figure 7C), coexpression of Fz2-GPI with Fz2 together activates *sens* up to seven to eight cells wide, which is three to four cells wider than those expressing Fz2 alone (Figure 7D). Therefore, similar to Dlp, Fz2 could convert Fz2-GPI from an inhibitor to an activator in Wg signaling.

DISCUSSION

The mechanisms controlling Wg signaling and its gradient formation are highly complex. Here, we have provided two lines of findings for the mechanistic roles of Dlp in Wg signaling. First, we show that the core protein of Dlp has similar biphasic activity to wild-type Dlp in Wg signaling. Consistent with this, the Dlp core protein can interact with Wg, while the attached HS chains can enhance Dlp's affinity for Wg binding. Second, we demonstrate that Dlp can get a biphasic response without Notum cleavage, and the ratio of Dlp:Fz2 determines its biphasic activity in cell culture and in the wing disc. While a low ratio of Dlp:Fz2 can help Fz2 obtain more Wg, a high ratio of Dlp:Fz2 prevents Fz2 from capturing Wg. We propose that the main activity of Dlp in Wg signaling is to retain Wg on the cell membrane rather than to act as a classic coreceptor. Dlp can mediate the exchange of Wg between receptors and itself; the net flow of the ligand depends on the ratios of the ligand, receptor, and Dlp. In support of our model, we found that Fz2-GPI also has biphasic activity in Wg signaling.

Mechanism of Dlp's Biphasic Activity in Wg Morphogen Signaling

Previous studies have demonstrated that Dlp acts as a biphasic modulator for Wg signaling in the wing disc; however, the mechanism underlying this biphasic response is not clear. One model suggests that Notum expressed at the D/V boundary can cleave Dlp and release it together with bound Wg, converting Dlp from a membrane coreceptor to a secreted antagonist (Kreuger et al., 2004). Our data suggest that this model needs to be revised. First, we show that expression of a GPI-deleted secreted form of Dlp (similar to the form cleaved by Notum) does not inhibit Wg signaling in the wing discs. Second, expression of CD2 forms of Dlp, which cannot be cleaved by Notum, can also inhibit *sens* expression similar to GPI versions of Dlp. An alternative model is that Dlp competes with Wg receptors on the cell surface, locally inhibiting signaling, but it also promotes long-range Wg gradient formation, and thus provides more Wg in the distal part of the wing disc (Franch-Marro et al., 2005; Hufnagel et al., 2006). However, this model cannot explain how Dlp has biphasic effects in vitro, where Wg gradients do not form (our results and those from Baeg et al. [2004]).

On the basis of our results, we favor an exchange factor model to explain the biphasic activity of Dlp in Wg signaling (Figure 7E). Our model is very similar to a recently published mathematical

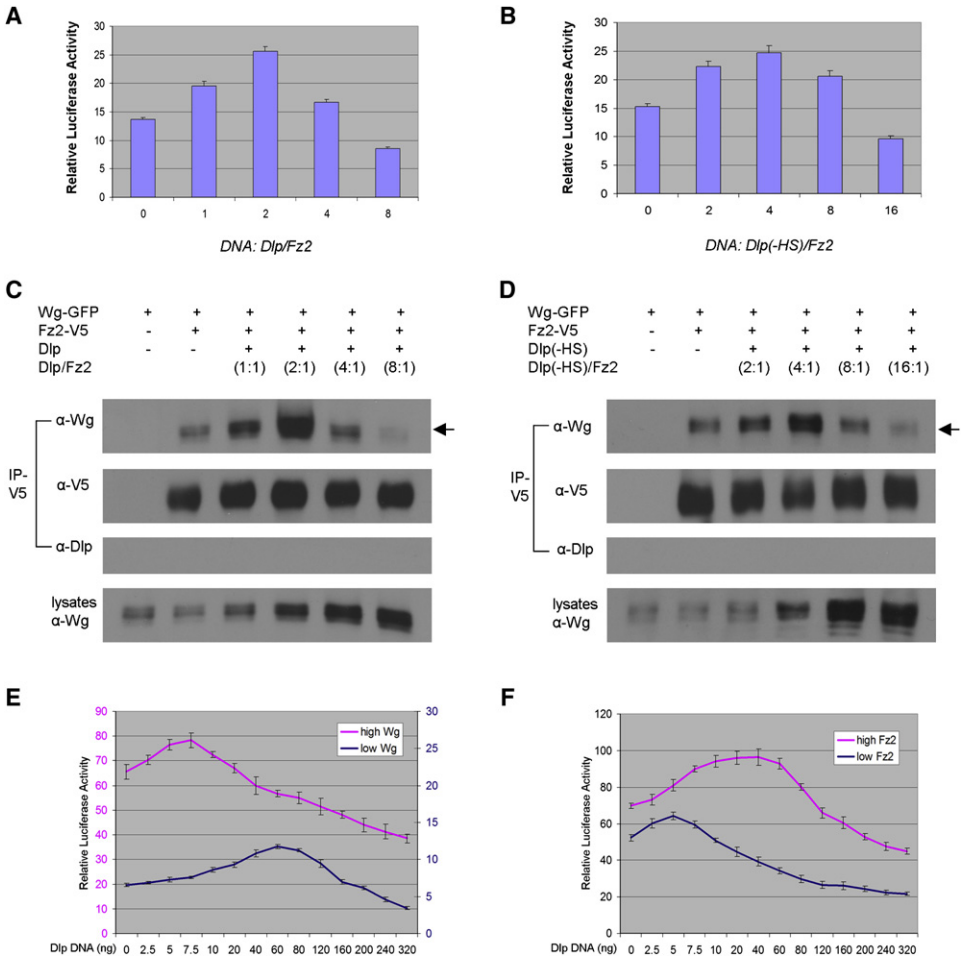

Figure 6. Fz2:Dlp Ratio Determines Dlp's Biphasic Activity in Wg Signaling

(A–B) S2 cells were transfected with the 12xdTOP-Luciferase reporter, the Renilla normalization vector, 20 ng Fz2 expression plasmids, variable amounts of Dlp or Dlp(−HS) expression plasmids, and then incubated with Wg-conditioned medium. (A) The columns represent Luciferase activities in the absence of Dlp or in the presence of Dlp with the Dlp/Fz2 DNA ratio of 1, 2, 4, and 8, as indicated. (B) Luciferase activities in the absence of Dlp(−HS) or in the presence of Dlp(−HS), with the Dlp(−HS):Fz2 DNA ratio of 2, 4, 8, and 16, as indicated. While a small amount of Dlp or Dlp(−HS) enhances Wg signaling, a large amount of Dlp/Dlp(−HS) inhibits Wg signaling. The error bars represent standard deviations.

(C–D) Fixed amounts of Wg-GFP or Fz2-V5 and variable amounts of Dlp or Dlp(−HS) expression vectors were transfected individually or together into S2 cells. Top three panels: cell lysates were immunoprecipitated and analyzed by Western blotting with the antibodies indicated. Bottom panel: the amount of Wg-GFP in 5% of cell lysates input was assessed by Western blot. A low level of Dlp or Dlp(−HS) helps Fz2 pull down more Wg, but a high level of Dlp or Dlp(−HS) reduces Wg coprecipitated by Fz2 (arrows). Note that the total amount of Wg in the lysates increases as more Dlp/Dlp(−HS) was added, probably reflecting its ability to stabilize Wg. Also, Dlp was not found coprecipitated with Fz2, suggesting that Dlp does not form a stable complex with Fz2 as a coreceptor. (E–F) Dlp's biphasic curve changes in different Wg and Fz2 concentrations.

(E) S2 cells were transfected with the Luciferase reporter, the normalization vector, 20 ng Fz2 expression plasmids, variable amounts of Dlp expression plasmids, as indicated, and then incubated with two different concentrations of Wg-conditioned medium. High Wg-conditioned medium is 10 times more concentrated than low-Wg medium. In the low-Wg condition, data are plotted on the right axis. In the high-Wg condition, the biphasic point shifts to the left.

(F) S2 cells were transfected with the Luciferase reporter, the normalization vector, 10 ng or 60 ng Fz2 expression plasmids, variable amounts of Dlp expression plasmids, as indicated, and then incubated with Wg-conditioned medium. In the high Fz2 condition, the biphasic point shifts to the right. The error bars represent standard deviations.

model for biphasic activity of CV-2 in BMP signaling (Serpe et al., 2008). In this model, Dlp might either compete with the receptor or provide ligands for the receptor, its role changing depending on the relative levels of ligand, receptor, and exchange factor. We show that, in the wing discs, raising the levels of Fz2 can convert Dlp from a repressor to an activator. In S2 cells, the biphasic activity of Dlp also depends on the Dlp:Fz2 ratio, with a low level of Dlp increasing Wg signaling reporter activity and a high level of Dlp reducing its activity. Using Co-IP experiments, we directly show that a small amount of Dlp provides Wg for Fz2 receptor, while a large amount of Dlp sequesters the Wg ligand. Moreover, we found that, for a constant amount of Dlp, it is more likely to repress Wg signaling at high Wg concentration, but to promote signaling at low Wg concentration. In contrast, Dlp is more likely to promote Wg signaling at high Fz2 concentration, but to repress signaling at low Fz2 concentration. Thus, our

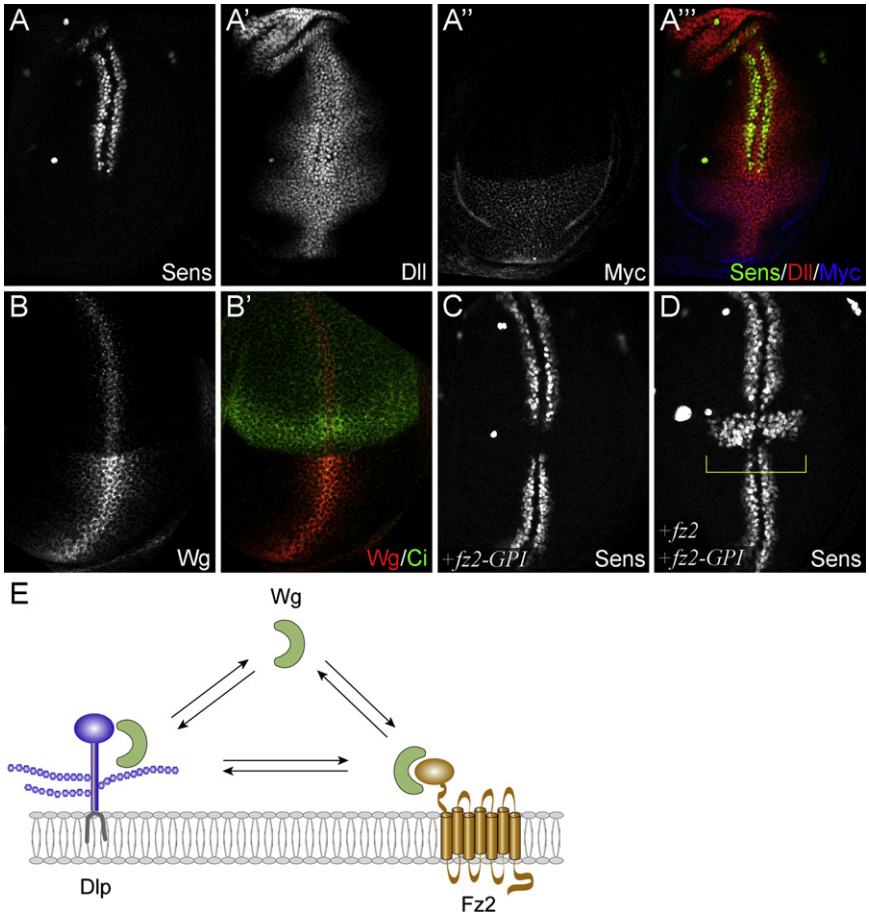

Figure 7. Fz2-GPI Has Biphasic Activity in Wg Morphogen Signaling

(A–A‴) Expression of Fz2-GPI in the posterior compartment by *hh-Gal4* eliminates *sens* expression (A) and expands *dll* expression domain (A′ and A‴). Fz2-GPI expression was detected by anti-Myc antibody, since it has a myc tag inserted (A″). Because persistent expression of Fz2-GPI by *en-Gal4* leads to greatly reduced posterior compartment, we used the Gal80ts technique and induced Fz2-GPI expression at 30°C for 16 hr prior to dissection.

(B–B′) Expression of Fz2-GPI in the posterior compartment by *hh-Gal4* causes Wg accumulation on the cell surface. The posterior part is marked by the absence of Ci staining.

(C–D) Expression of Fz2-GPI by *dpp-Gal4* inhibits *sens* expression (C). However, coexpression of Fz2 with Fz2-GPI leads to ectopic activation of *sens* up to seven to eight cells (D), which is three to four cells wider than expression of Fz2 alone.

(E) The exchange factor model for Dlp's role in Wg signaling. Dlp can provide Wg for the signaling receptors by retaining Wg on the cell surface; it can also compete with the receptor for ligand binding. As such, the net flow of Wg depends on the relative levels of Dlp, Wg, and Fz2.

model could explain the situation in wing disc, where Dlp inhibits Wg signaling in regions close to the D/V border (high Wg and low Fz2), and promotes signaling in regions far from the D/V border (low Wg and high Fz2). These data are consistent with a previous reports by Baeg et al. (2004), showing that in vitro Dlp promotes Wg signaling when the Wg level is low, but reduces signaling when the Wg level is high. This result also fits well with the theoretical modeling data of Serpe et al. (2008) for different ligand levels, suggesting Dlp acts similarly to CV-2 in different systems. In order to work, their model contains a tripartite complex between CV-2, BMP, and the receptor. We did not detect Dlp coprecipitated with Fz2; however, as they proposed, the intermediate is a transient complex with very rapid on-off kinetics, and it is difficult to demonstrate the tripartite intermediate directly. Finally, in further support of our model, we found that Fz2-GPI, which can stabilize Wg on the cell surface and compete with Fz2 for Wg binding, also has biphasic activity in Wg signaling.

Previous studies reported that secreted Fz-related protein (sFRP), another family of Wnt-interacting proteins, can also exhibit biphasic activity in Wnt signaling, enhancing Wnt signaling at low concentration, but inhibiting it at high concentration (Uren et al., 2000). As mentioned above, the BMP-binding protein, CV-2, can act as a concentration-dependent, biphasic regulator for BMP signaling in the wing disc (Serpe et al., 2008). It is interesting to note that both sFRP and CV-2 can interact with HSPGs, and are likely to exert their function on the cell surface (Serpe et al., 2008; Uren et al., 2000). In addition, another HSPG member, *Xenopus* Syndecan-1, shows a level-dependent activation or inhibition of BMP signaling during dorsoventral patterning of the embryonic ectoderm (Olivares et al., 2009). Moreover, we found that Ihog, a recently identified Hh coreceptor (Yao et al., 2006), has biphasic activity in Hh morphogen signaling. Overexpression of Ihog represses high-threshold Hh target, and extends low-threshold Hh target gene expression (D.Y., Y.W., X.L., unpublished data). Together, other cell surface ligand-interacting proteins might regulate signaling by a similar mechanism. Traditionally, all cell surface ligand-binding receptors that cannot signal independently are equivocally called coreceptors, despite their diverse functions. On the basis of our results, we propose that some of the coreceptors may function as the exchange factors rather than the classical coreceptors, which only enhance signaling by providing ligand to the receptor.

Dlp Core Protein Can Interact with Wg Independent of Its GAG Chains

Another important finding of this work is the demonstration that Dlp's major activity in Wg signaling depends on its core protein. Previous studies have shown that different HSPG proteins play very distinct roles in Wg signaling and distribution (Lin, 2004). However, the mechanism underlying this specificity is unknown. Here, we present evidence that the specificity of Dlp in Wg signaling results from its core protein. First, the Dlp core protein has biphasic activity for short- and long-range signaling similar to that of wild-type Dlp. Second, the Dlp core protein interacts with

Wg in co-IP experiment, cell-binding assay, as well as in the wing discs. Third, we show that the N-terminal domain of Dlp is essential for Wg binding, and that fusion of the N-terminal domain of Dlp to the Fz2 membrane and cytoplasmic domain can recapitulate Fz2 activity. These data are consistent with previous results indicating that *Xenopus* glypican-4 interacts with Wnt11 through its N-terminal domain (Ohkawara et al., 2003). It is interesting to note that, similar to Fz2 CRD domain, the N-terminal domain of Dlp protein has 14 highly conserved cysteines, a shared feature of all glypican members (Baeg et al., 2001; Filmus et al., 2008).

Previously, Filmus and colleagues showed that vertebrate glypican-3 core protein is directly involved in Wnt signaling, whereas the GAG chains of glypican-3 are not required for the stimulatory effect in Wnt signaling (Capurro et al., 2005). Moreover, their recent data show that the glypican-3 core protein also binds to Sonic Hedgehog (Shh), but inhibits its signaling by competing with the receptor, Patched (Capurro et al., 2008). The opposite effects of the same glypican core protein on Wnt and Hh signaling are intriguing. Interestingly, we also observed that the Dlp core protein positively regulates Hh signaling in both *Drosophila* embryos and wing discs (D.Y., Y.W., X.L., unpublished data). Thus, the core proteins of glypican-3 and Dlp appear to have opposite roles in Wnt and Hh signaling (Beckett et al., 2008; Yan and Lin, 2008). It is likely that different glypican cores may bear distinctive motifs to interact with Wnt and Hh proteins.

Although the Dlp core protein is able to bind Wg, we found that the attached HS GAG chains are also important for the binding affinity between Dlp and Wg. Wild-type Dlp shows significantly stronger binding for Wg than the core protein alone. This result is consistent with previous genetic experiments showing that Wg signaling is compromised in HS-deficient mutants. In addition, biochemical studies also suggest that Wg is a heparin-binding protein (Reichsman et al., 1996). One possibility is that the Dlp core protein might have different membrane distribution than wild-type Dlp, as previously reported (Mertens et al., 1996). However, we did not observe obvious difference in the subcellular localizations between Dlp-GFP and Dlp(−HS)-GFP (Figures S4D–S4F″). It remains to be determined how the presence of HS GAG chains can enhance Dlp's ability to bind Wg.

The GPI Anchor of Dlp Is Not Essential for Its Activity in Wg Signaling

All glypicans anchor to the cell membrane via a GPI anchor (Bernfield et al., 1999; Lin, 2004). GPI proteins are enriched in specific membrane subdomains called lipid rafts, which are suggested to promote the signaling activities of GPI-anchored proteins (Mayor and Riezman, 2004). Thus, one important issue is whether the GPI anchor is required for Dlp's activity in Wg signaling. Our results suggest that the GPI anchor of Dlp is not essential for its activity in Wg signaling. Several lines of evidence support our view. First, Dlp(−HS)-CD2, a transmembrane form of Dlp core protein, has similar biphasic activity to that of Dlp(−HS). Second, we analyzed the subcellular localizations of different forms of Dlp, and found that Dlp's major activity is to bind Wg at the cell surface. Dlp-GFP, which has the strongest binding affinity for Wg, accumulates more Wg on the cell surface. In Dlp(−HS)-GFP and Dlp(−HS)-CD2-GFP-expressing discs, we found less Wg accumulated on the cell membrane and more internalized Wg vesicles. Our results are consistent with a recent work showing that accumulating Wg on Dlp-expressing cells is less accessible to internalization (Marois et al., 2006). Although Dlp-GFP and Dlp(−HS)-GFP, but not Dlp(−HS)-CD2-GFP, form many endocytic vesicles due to a role of the GPI anchor in trafficking, based on our functional data, we suggest that the GPI anchor of Dlp is not essential for Wg signaling.

Recently, Therond and his colleagues proposed that the GPI anchor of Dlp is required for Wg internalization and long-range signaling (Gallet et al., 2008). This conclusion is mainly based on the evidence that expression of their GFP-Dlp-CD2 can reduce the expression of Wg long-range target gene *dll*. This result is apparently different from our data showing that expression of Dlp(−HS)-CD2-GFP construct leads to expanded *dll* expression. To resolve this issue, we obtained the GFP-Dlp-CD2 transgenic flies used by Gallet et al. (2008), and examined the activity of GFP-Dlp-CD2 in the wing discs. We have observed different results from the data described by Gallet et al. (2008). We found that their GFP-Dlp-CD2 has very similar biphasic activity to our Dlp-GFP when it is expressed by *en-Gal4* or *ap-Gal4*, and sought to observe the effects on *dll* expression (Figures 4A–4B″). One possibility for the difference is that they only use *ap-Gal4*, which will cause expression of Dlp to reduce the size of the compartment; this may complicate comparisons of the effect of GFP-Dlp-CD2 in long-range signaling. We therefore chose to use *en-Gal4*, which allows the use of the A compartment as an internal control. Furthermore, while they showed that GFP-Dlp-CD2 induces a more severe wing defect than their GFP-Dlp construct, we found that GFP-Dlp-CD2 does not generate a more severe wing defect than our Dlp-GFP construct (Baeg et al., 2004) (Figures 4E and 4F). In this regard, it is important to note that the GFP-Dlp-CD2 and GFP-Dlp constructs used by Gallet et al. (2008) employed GFP inserted at two different sites in Dlp, and that the insertion in the GFP-Dlp construct leads to reduced activity (Han et al., 2004b) (Figure 4G). In conclusion, our data demonstrate that CD2 forms of Dlp have similar activity to the GPI forms of Dlp, suggesting that the GPI anchor of Dlp is not essential for its activity in Wg signaling.

EXPERIMENTAL PROCEDURES

Drosophila Strains and Plasmid Construction

See the Supplemental Data For information on *Drosophila* strains and plasmid construction.

Antibodies and Immunofluorescence

The wing disc staining procedure was preformed as previously described (Han et al., 2004b). The following primary antibodies were used: mouse anti-Dll (Duncan et al., 1998), guinea pig anti-Sens (Nolo et al., 2000), mouse anti-Wg (4D4; DSHB), rabbit anti-GFP Alexa Fluor 488 (Molecular Probes), mouse anti-Dlp (Lum et al., 2003), rabbit anti-Dlp (Baeg et al., 2001), rabbit anti-V5 (Sigma), mouse anti-ΔHS 3G10 (Seikagaku Corporation), rat anti-Ci (Motzny and Holmgren, 1995), rabbit anti-Myc (Cell Signaling), and rabbit anti-Fz2 (Mathew et al., 2005). To detect HS, the wing discs were dissected and fixed, then treated with 500 mU/ml heparinase III (Sigma) in 37°C for 6 hr and stained with 3G10 antibody. For dextran labeling, the wing discs were incubated in 0.25 mM Texas red dextran (lysine fixable, MW3000; Molecular Probes) in M3 medium at 25°C for 10 min pulse, followed by five times 2 min washes in ice-cold M3 medium. After that, discs were chased for 20 min at 25°C in M3 medium, then fixed and processed per standard procedure (Piddini et al.,

2005; Rives et al., 2006). After the chase period, most Texas red dextran is internalized and present in endocytic compartments; only a residual level of dextran remains on the cell membrane. The primary antibodies were detected by Cy3, Cy5-conjugated (Jackson Immuno), or Alexa Fluor 488-conjugated (Molecular Probes) secondary antibodies. The primary antibodies used for IP and Western blot were guinea pig anti-Dlp (made in our laboratory), rabbit anti-V5 (Sigma), guinea pig anti-GFP (made in our laboratory), mouse anti-V5 (Invitrogen), mouse anti-Wg (4D4; DSHB). For image quantification in Figure S1, the raw data of antibody staining were exported in tiff file format. The fluorescence values were measured from selected regions in Image J using plot profile function. The plot values were then used to generate plot profiles in Microsoft Excel. To quantify vesicles in Figure 3, images were analyzed in Image J by the threshold function, and Wg expression cells were excluded. We then used the "analyze particle" function to count particles as a signal of at least three contiguous pixels (Marois et al., 2006). We averaged five discs for each condition.

Cell-Binding Assay and Coimmunoprecipitation

Drosophila S2 cells were grown on coverslips and transiently transfected with various *dlp* constructs using Effectene (QIAGEN). After 24 hr, the cells were incubated in Wg-conditioned medium for 3 hr on ice, then fixed and stained with Wg and other antibodies indicated in the figures (Bhanot et al., 1996; Franch-Marro et al., 2005). For Co-IP experiments in Figure 2H, S2 cells were transfected in 100 mm dishes with 4 μg total DNA, including *pUAST-dlp* (or other dlp construct), *pAc-wg-GFP*, and *pArmadillo-Gal4*. For Co-IP experiments in Figures 6C–6D, S2 cells were transfected in 100 mm dishes with 6 μg total DNA, including *pUAST-fz2-V5*, *pUAST-dlp/pUAST-dlp(−HS)*, *pAc-wg-GFP*, and *pArmadillo-Gal4*. The ratios of *dlp/dlp(−HS)* to *fz2* are indicated in the figures. Cells were harvested 60 hr later and lysed in 900 μl of 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 2% Triton X-100, 1 mM EDTA, plus proteinase inhibitors (Roche) on ice for 1 hr. After preclearance with protein G Sepharose 4 Fast Flow (Amersham) beads, the lysate was incubated with antibodies for 4 hr at 4°C, and then incubated for an additional 2 hr in the presence of 25 μl of beads. Beads were washed four times with 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.2% Triton X-100, 1 mM EDTA, and eluted in Laemmli sample buffer. Western blotting was conducted as previously described (Han et al., 2004a).

Luciferase Reporter Assay

Transfections were performed in 24 well plates by using Effectene transfection reagent in S2 cells. In each well, 450 ng of total DNA was added, including 12XdTOP Luciferase reporter, PolIII-RL normalization vector (DasGupta et al., 2005), *pUAST-fz2*, and *pUAST-dlp* or *pUAST-dlp(−HS)*. The amounts of *fz2* and *dlp* plasmids are indicated in the figures. After 48 hr, concentrated Wg-conditioned medium was applied on cells for an additional 20 hr. Cells were then lysed, and Luciferase activities were measured using Dual-Luciferase Assay kits (Promega).

SUPPLEMENTAL DATA

Supplemental Data include four figures and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00383-9.

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