Opposing Activities of Dally-like Glypican at High and Low Levels of Wingless Morphogen Activity

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Summary

The glypican family of heparan sulfate proteoglycans has been implicated in formation of morphogen gradients. Here, we examine the role of the glypican Dally-like protein (Dlp) in shaping the Wingless gradient in the *Drosophila* wing disc. Surprisingly, we find that Dlp has opposite effects at high and low levels of Wingless. Dlp promotes low-level Wingless activity but reduces high-level Wingless activity. We present evidence that the Wg antagonist Notum acts to induce cleavage of the Dlp glypican at the level of its GPI anchor, which leads to shedding of Dlp. Thus, spatially regulated modification of Dlp by Notum employs the ligand binding activity of Dlp to promote or inhibit signaling in a context-dependent manner. Notum-induced shedding of Dlp could convert Dlp from a membrane-tethered coreceptor to a secreted antagonist.

Introduction

Proteoglycans carrying glycosaminoglycan (GAG) side chains play important roles as modulators of the activity of secreted signaling molecules. Most secreted signaling proteins have been shown to bind directly to isolated GAGs, in the form of heparin or heparan sulfate, or to intact heparan sulfate proteoglycans (HSPG; reviewed in Perrimon and Bernfield, 2001; Selleck, 2001). HS chains are long unbranched polymers, assembled from alternating N-acetyl-glucosamine and glucuronic acid units, that undergo sequential modification in the Golgi to generate highly anionic final structures rich in iduronic acid and sulfate. HS chains are thought to contain diverse sulfated epitopes that constitute binding sites for protein ligands (Gallagher, 2001).

In some cases, HSPGs are thought to function as coreceptors, contributing to formation of active signaling complexes. The best evidence for an involvement of GAG in ligand-receptor interaction is for fibroblast growth factors (FGF). Crystal structures of a ternary complex between FGF, its cognate FGF receptors, and heparin oligosaccharides provide direct evidence for a contribution of a GAG to ligand binding (Schlessinger, 2000; Pellegrini et al., 2000). It has also been shown that FGFs bind selectively to sequences in HS that are rich in sulfate, and there is evidence that the dynamic expression of such sequences modulates the interaction between the ligand and its high-affinity receptor to impact

upon downstream signaling (Nurcombe et al., 1993; Lundin et al., 2000; Kreuger et al., 2001). GAGs are thought to mediate Wg activity because Wg is released into the culture medium from Wg-producing cells by addition of GAG and because Wg can bind to heparin (Reichsman et al., 1996). In addition, Wg binding to imaginal disc cells can be reduced by treatment with GAG-degrading enzymes (Greco et al., 2001). Genetic studies in *Drosophila* have shown that GAG biosynthesis is essential for normal FGF, Wingless, and Hedgehog activity. Mutants defective in GAG biosynthesis are impaired in signaling by and/or movement of these ligands (Hacker et al., 1997; Binari et al., 1997; Bellaiche et al., 1998; Haerry et al., 1997; The et al., 1999; Lin et al., 1999; Lin and Perrimon, 1999; Goto et al., 2001; Selva et al., 2001). Qualitative aspects of GAG sulfation also play an important role for these signaling activities (e.g., Dhoot et al., 2001; Ai et al., 2003; Esko and Lindahl, 2001).

Glypicans are an important class of core protein to which GAG side chains are added to make HSPGs. Glypicans act as mediators of Wnt, FGF, and BMP activity in vertebrate embryos (Galli et al., 2003; Latinkic et al., 2003; Paine-Saunders et al., 2002; Topczewski et al., 2001; reviewed in Song and Filmus, 2002). Mutations in the human glypican GPC3 gene cause Simpson-Golabi-Behmel syndrome, which is characterized by pre- and postnatal overgrowth (Pilia et al., 1996). In *Drosophila*, mutants that reduce activity of the glypican Dally show impaired Wg, Hh, and Dpp signaling activity and gradient formation in vivo (Jackson et al., 1997; Lin and Perrimon, 1999; Tsuda et al., 1999; Fujise et al., 2001, 2003). It has been suggested that Dally might serve as a coreceptor mediating ligand receptor interaction (Lin et al., 1999; Tsuda et al., 1999; Fujise et al., 2003). A second glypican called Dally-like (Dlp) has been implicated in Wg signaling based on RNAi treatment in embryos and overexpression in imaginal discs (Baeg et al., 2001). Other studies have suggested that reduction of Dlp levels by RNAi has a stronger effect on Hedgehog than on Wg signaling, questioning whether Dlp plays a role in Wg signaling in the embryo (Desbordes and Sanson, 2003; Lum et al., 2003). However, as these treatments do not eliminate Dlp, a role in Wg signaling cannot be excluded.

Here, we present evidence that Dlp plays a role in Wg signaling in the wing imaginal disc. Dlp acts as a positive cofactor to increase Wg activity where Wg levels are low. Paradoxically, Dlp reduces Wg activity in cells close to the source of Wg production, where Wg levels are highest. This apparent discrepancy may be explained by the activity of the secreted protein Notum, which serves as a Wg antagonist (Giraldez et al., 2002; Gerlitz and Basler, 2002). Notum expression is locally induced by high-level Wg activity. We present evidence that Notum induces cleavage of Dlp to release it from its GPI anchor. We propose that spatially regulated cleavage of Dlp by Notum is responsible for the different activities of the Dlp glypican in different regions of the Wg morphogen gradient.

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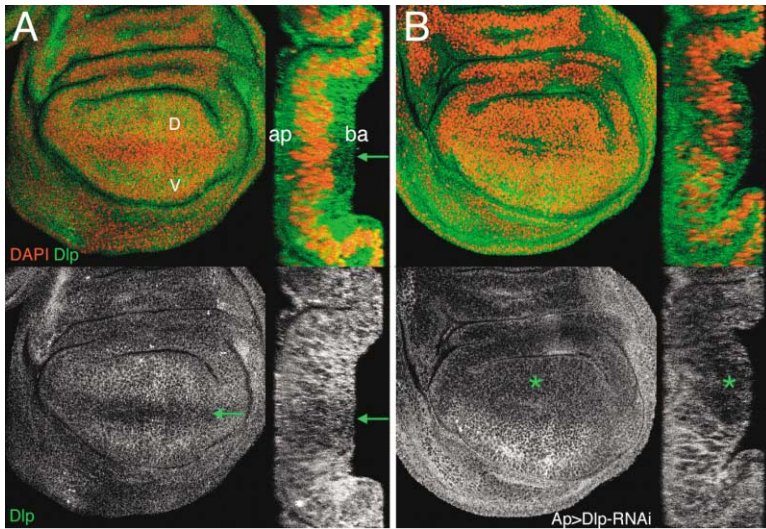
Figure 1. Dlp Expression in the Wing Disc

Wing disc labeled with antibody to Dlp protein (green). Nuclei labeled with DAPI (red). Optical cross-sections of the discs are shown at right. Abbreviations: ap, apical; ba, basolateral. The Dlp channel is shown separately below. D and V indicate dorsal and ventral compartments of the wing.
(A) Wild-type disc showing reduced Dlp levels in the center of the wing pouch (green arrow).
(B) Disc expressing a *UAS-Dlp-RNAi* construct in D cells under *apGal4* control. Note the reduced level of Dlp protein in D cells (e.g., asterisk).

Results

Dlp Has Positive and Negative Effects on Wg Signaling

Initial reports implicated Dally and Dlp in Wg signaling in the embryo (Baeg et al., 2001; Lin and Perrimon, 1999; Tsuda et al., 1999). Another report suggests that Dlp is required for Hh signaling but not for Wg signaling in the embryo and that Dally is not required for either (Desbordes and Sanson, 2003). A role for Dlp in Hh but not Wg signaling is supported by RNAi-based cell culture assays (Lum et al., 2003). On the other hand, Dally mutants showed reduced Wg activity in the wing (Lin and Perrimon, 1999), and overexpression of Dlp can compromise Wg gradient formation in the wing disc (Baeg et al., 2001). If Dlp is not needed for Wg signaling per se, it seemed likely that its role might be in modulating Wg gradient formation. As a first step to evaluate Dlp function, we prepared an antibody to Dlp protein and examined its expression in the wing disc. Dlp protein levels are low near the source of Wg production and increase in more distant cells (Figure 1A). An optical cross-section suggests that the endogenous Dlp protein is more abundant on the basolateral cell surface (Figure 1A), where the extracellular Wg gradient forms (Strigini and Cohen, 2000). To verify that the antibody accurately visualizes endogenous Dlp expression, we prepared a UAS-Dlp-RNAi construct, which permits GAL4-dependent expression of double-stranded Dlp RNA leading to RNA interference. Expression of this construct in dorsal cells under *ap-Gal4* control reduced the level of Dlp protein visualized by antibody labeling (Figure 1B), confirming that the antibody accurately reflects Dlp expression.

We next made use of RNAi to reduce Dlp activity in vivo. When coexpressed with UAS-Dlp, the RNAi construct effectively suppressed the phenotype produced by Dlp overexpression phenotype (not shown), confirming that UAS-Dlp-RNAi is effective in vivo. The effects of reduced Dlp expression on Wg morphogen activity was examined using antibodies to Vestigial and Distalless, two Wg targets (Neumann and Cohen, 1997; Zecca et al., 1996). Vestigial expression is high near the source of Wg and decreases in a graded fashion toward dorsal and ventral (Figure 2A). The intensity of Vestigial expression was lower in the dorsal compartment of discs with reduced dorsal Dlp expression, and the range over which Vestigial was expressed was reduced (Figure 2B). In addition, the size of the dorsal compartment was smaller than normal, as was observed when Wg signaling activity was reduced in dorsal cells (Giraldez and Cohen, 2003). The effect on Distalless expression was similar, but more variable. These observations suggest a positive role for Dlp in promoting low-level Wg activity and are consistent with the observation that Dlp protein levels are higher in the part of the disc where Wg signaling levels are lower.

Surprisingly, we obtained an opposite effect when we examined the effects of reduced Dlp on a high-threshold Wg target. High levels of Wg signaling specify the domain in which the wing margin sense organs form (Couso et al., 1994), visualized by expression of the transcription factor Senseless (Figure 2C; Nolo et al., 2000). Reduction of Dlp in the wing pouch caused a modest but consistent increase in the width of the Senseless protein domain (Figure 2D; the spacing between D and V subdomains was reproducibly irregular). This resulted in an excess of bristles in the anterior wing margin (Figures 2E–2G). Wild-type wings have a single row of mechanosensory bristles and a second row in which a chemosensory bristle appears in every fourth position along the anterior margin on the dorsal wing. Figure 2G shows the number of additional bristles in wings with reduced Dlp expression compared to control wings. Increased bristle number can reflect an increase in Wg activity (e.g., Axelrod et al., 1996; Zhang and Carthew, 1998). Thus, reduction of Dlp activity leads to increased Wg activity in cells close to the wing margin, where Wg levels are highest. Although this is where Dlp protein levels are normally low, further reducing Dlp leads to a local increase in Wg activity.

These findings lead to the surprising conclusion that Dlp can promote Wg activity where ligand levels are low and that it can reduce Wg activity where ligand levels are high. Binding of Wg to Dlp could help to retain ligand near the cell surface and directly or indirectly facilitate
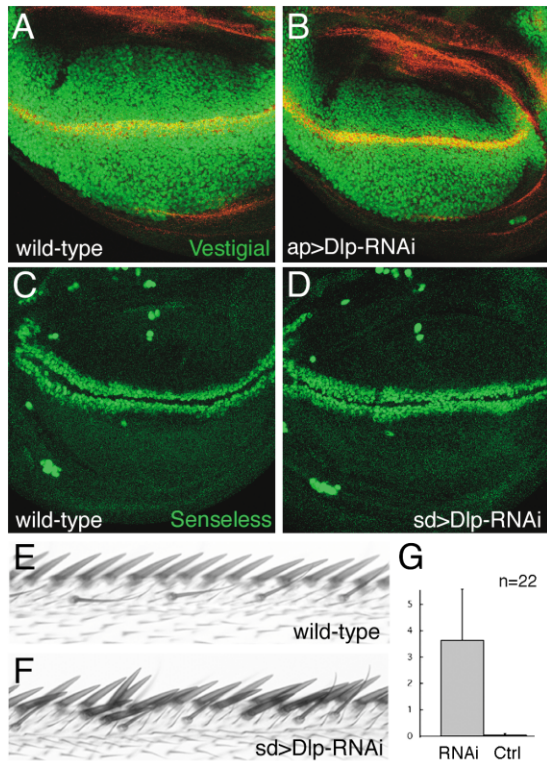
Figure 2. Dlp RNAi Phenotypes

(A and B) Wing discs labeled to visualize Vestigial protein (green) and Wingless protein (red).

(A) Wild-type disc. Note the symmetry of Vestigial levels in D and V compartments.

(B) *apGal4 UAS-Dlp-RNAi* disc. Note the reduced brightness of Vestigial in the D compartment compared to the V compartment and the smaller size of the D compartment compared to the V compartment in the same disc. The wing pouch is also smaller overall.

(C and D) Senseless protein expression (green) in wild-type (C) and *sd-GAL4; UAS-Dlp-RNAi* wing discs (D). Note that the spacing between the dorsal and ventral Senseless domains was irregular in the RNAi-expressing discs (D). These domains are normally clearly separated, so it is noteworthy that the irregularity was highly reproducible.

(E) Wild-type anterior wing margin in the dorsal compartment. Note the single well-organized row of stout mechanosensory bristles and the thin curved chemosensory bristle in every fourth position.

(F) Detail of the anterior margin of an *sd-GAL4; UAS-Dlp-RNAi* wing showing extra mechanosensory and chemosensory bristles.

(G) Quantification of extra mechanosensory bristles per wing in the anterior wing margin in *sd-GAL4; UAS-Dlp-RNAi* and control w^{1118} flies. n = 22 for both groups, t test p < 1E−10.

presentation of the ligand to the receptor complex. This could increase signaling activity where ligand levels are low. The antagonistic effects of elevated glypican expression might be explained if binding to the glypican sequesters ligand and limits its availability for binding to the signaling receptor. However, this view of glypican function does not provide a satisfactory explanation for the observation that reduced Dlp causes an increase in Wg activity near the dorsoventral boundary of the wing disc.

Notum Acts on Reduced-GAG Dlp

Previous studies have identified Notum as a Wg antagonist. Loss of Notum activity caused an increase in Wg

protein levels and an increase in the number of wing margin bristles (Figure 2 in Giraldez et al., 2002), which closely resembles the Dlp-RNAi phenotype. This prompted us to reexamine how Notum acts on Dlp.

Notum encodes a secreted member of the α/β-hydrolase superfamily, which includes serine proteases, lipases, and other enzymes in which a Ser-Asp-His catalytic triad comprises the active site (Nardini and Dijkstra, 1999). Mutation of Ser237 to Ala was shown to remove Notum activity in vivo and in vitro (Giraldez et al., 2002). On the basis of its similarity to pectin acetylesterase enzymes from plants and plant pathogens and on the basis of its ability to compete with the N-deacetylase-N-sulfotransferase enzyme for use of Dlp as a substrate, we previously proposed that Notum might function as a GAG deacetylase. If this were the case, we would expect Notum to be unable to modify a form of Dlp lacking GAG side chains.

Dally and Dlp each contain five putative GAG addition sites (Figure 3A) and have been shown to carry predominantly heparan sulfate (HS; Tsuda et al., 1999; Baeg et al., 2001; Lin et al., 1999), as shown for vertebrate glypicans (Chen and Lander, 2001). GAG biosynthesis is initiated by addition of a xylose residue to the hydroxyl group of a serine in a serine-glycine (SG) motif in the stem region of the glypican (Lindahl et al., 1998). We prepared mutant forms of Dally and Dlp proteins in which all the putative GAG-addition sites were mutated. For Dally, the serine residues in the five SG motifs were mutated to alanine. For Dlp, four SG motifs were deleted by removing a block of 25 residues, and the fifth was mutated to AG.

The glycosylation states of epitope-tagged Dally and Dlp and the GAG addition site mutants were compared by anion exchange chromatography. Glypicans isolated from mammalian cells in culture are highly negatively charged and are typically retained on a strong anion exchange matrix up to 1 M NaCl (Ding et al., 2002). When expressed in Schneider S2 cells, a considerable fraction of Dally bound to Q-Sepharose in 0.3 M salt and some was able to bind in up to 1 M salt (Figure 3B). Dally migrated as a broad band in SDS-PAGE. The more negatively charged forms that were bound to Q-Sepharose at higher salt exhibited a higher apparent molecular weight. This may reflect the presence of more and longer HS side chains as well as differences in charge due to sulfation. Dally behaves like a conventional glypican (Tsuda et al., 1999). In contrast, most of the Dlp protein bound to Q-Sepharose in 0.15 M salt, but none bound at higher salt concentrations. Dlp migrated as a more tightly resolved band in SDS-PAGE. These observations suggest that Dally and Dlp differ in the extent or quality of GAG modification. Removal of the GAG addition sites considerably reduced retention of both mutant proteins on Q-Sepharose. The proportion of the mutant form of Dally bound was reduced at all salt levels and none was bound above 0.3 M salt. Very little of the mutant form of Dlp was able to bind to Q-Sepharose, even in physiological salt.

To further evaluate their GAG content, lysates of S2 cells transfected to express wild-type or mutant Dally or Dlp were digested with heparitinase, and the remaining glycan stub was detected with 3G10 antibody. Dally-expressing cells showed elevated GAG labeling in a
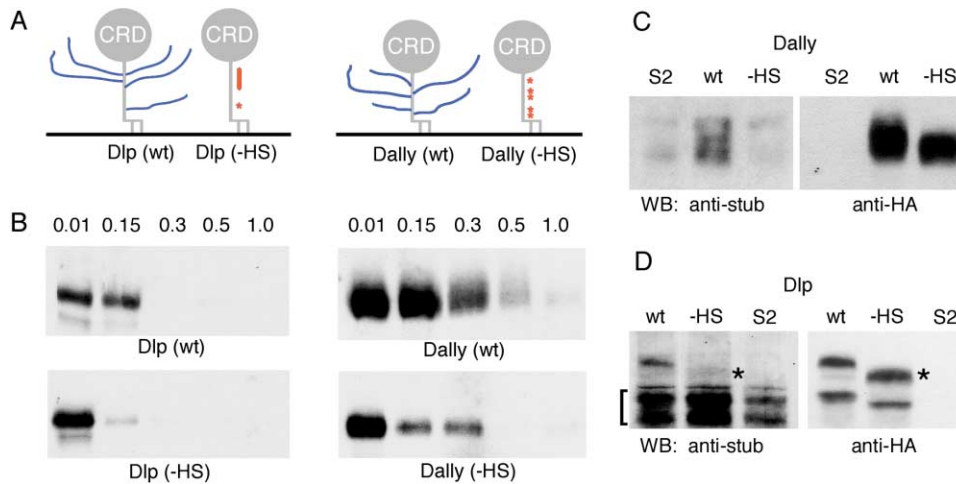
Figure 3. GAG Mutant Forms of Dally and Dlp

(A) Schematic representation of the endogenous and GAG-free forms of Dlp and Dally proteins. Wild-type (wt) Dally and Dlp proteins have multiple predicted SG GAG addition sites. GAG side chains are represented in blue. CRD denotes the globular cysteine-rich domain. (-HS) indicates the mutated proteins that lack the predicted GAG-attachment sites. Red stars represent replacement of SG GAG addition sites by AG. For Dally, five SG sites were mutated. For Dlp, the red bar represents a 75 bp deletion that removed four SG sites. The fifth SG was mutated to AG. All proteins were HA tagged.

(B) The degree of GAG modification of HA-tagged (wt) and (-HS) versions of Dlp and Dally was assessed by comparing their retention by anion exchange media. Lysates from S2 cells transfected to express the proteins were incubated with Q-Sepharose ion exchange resin pre-equilibrated in 10 mM NaPO$_4$ (pH 6) containing 0, 0.14, 0.3, 0.5, or 1.0 M NaCl, and then washed with 10 volumes of that buffer. Protein bound to the resin was eluted with 50 μl 3 M NaCl and loaded onto a SDS-PAGE gel. Blots were probed with rat anti-HA. The difference in Q-Sepharose binding of Dally and Dlp lacking GAG addition sites might be due to N-glycosylation or to differences in negatively charged amino acid residues present in the glypican core protein.

(C and D) Immunoblots of S2 cells transfected to express wild-type and GAG addition site mutant versions of Dally-HA (C) or Dlp-HA (D). Lysates were digested with Heparitinase I. Blots were probed with 3G10, which recognizes the glycan stub remaining after digestion of the GAG side chain, and with anti-HA. Bracket in (D) (left panel) indicates endogenous GAGs detected by anti-stub antibody, which are present in untransfected S2 cells. Asterisks indicate the Dlp-HS band.

broad band that comigrated with the major form of endogenous glypican in untransfected cells (Figure 3C). Although the level of expression of the mutant form of Dally was comparable (Figure 3C, anti-HA right panel), GAG labeling was not detectable above background (left panel). The level of GAG modification on overexpressed Dlp was lower than that seen on the endogenous glypicans (Figure 3D, bracket). This indicates that Dlp has a lower GAG content than Dally, consistent with its poor retention on Q sepharose. The mutant form of Dlp showed much reduced GAG labeling, barely distinguishable from background levels (Figure 3D, left panel, asterisk).

Dally resembles a conventional glypican, being extensively modified by GAG side chains. Dlp appears to have less heterogeneous and less extensive GAG modification than Dally. The mutant form of Dlp has low levels of residual GAG and would be expected to be a poor substrate for Notum, if Notum acts directly on the GAG side chains. Coexpression of Notum and HA-tagged Dlp in S2 cells caused a large shift in the electrophoretic mobility of Dlp in SDS-PAGE to a faster migrating species, and the Notum S237A mutant had no effect (Figure 4A, lanes 1 and 2). S2 cells contain endogenous Notum, so there is always a background level of Dlp processing (lane 3). Interestingly, Notum caused a shift in the mobility of the low-GAG form Dlp comparable in magnitude to the shift in the wild-type protein (Figure 4A, lane 4), though the processing efficiency was lower. This would

be difficult to explain if the shift were due to alteration in the amount or in the negative charge of the remaining GAG due to deacetylation. Further, we did not detect any increase in deacetylase activity (Pettersson et al., 1991) in lysates of S2 cells overexpressing Notum, nor was there any detectable decrease in the degree of HS acetylation (Toyoda et al., 2000) in larvae overexpressing Notum (not shown). These observations suggest that Notum does not act as a GAG-modifying enzyme.

Notum Induces Cleavage of Dlp
The mobility shift of Dlp caused by Notum is ~15 kDa. Proteolytic cleavage at the N or C termini of Dlp could cause the apparent size reduction, although other modifications such as removal or attachment of lipids, sugars, or phosphate groups also could cause anomalous migration in SDS-PAGE. To test the possibility of proteolytic processing, we prepared forms of Dlp with epitope tags close to the two ends. For Dlp-HA-C, an HA epitope tag was inserted at serine 732, just before the putative cleavage site for addition of the GPI anchor (S733). To produce GFP-Dlp-HA-C, the HA tag was placed at the corresponding position in GFP-Dlp, which contains a GFP moiety inserted in place of residue G68, close to the N terminus of the protein following removal of the signal peptide at residue 41. S2 cells were transfected with Dlp-HA, Dlp-HA-C, or GFP-Dlp-HA-C together with normal or mutant forms of Notum. Notum induced comparable mobility shifts in all three forms of Dlp (Figure
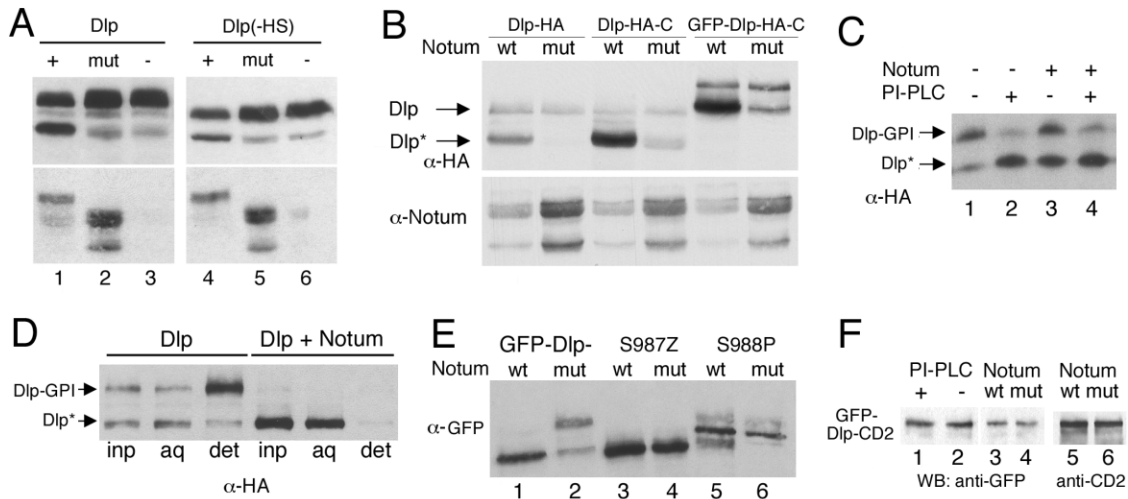
Figure 4. Processing of Dlp by Notum

(A) Notum acts on reduced-GAG Dlp. Immunoblots of S2 cells transfected to express Dlp or Dlp(-HS) with Golgi-tethered, wild-type Notum protein (+), the Notum S237A mutant (mut), or empty vector (−). Wild-type Dlp and Dlp(-HS) were both modified by Notum to increase the amount of the faster migrating form Dlp*.

(B) Immunoblot of S2 cells transfected to express Dlp-HA, Dlp-HA-C, and GFP-Dlp-HA-C with wild-type or mutant versions of Notum. Upper panel probed with anti-HA, lower panel with anti-Notum. The proportion of Dlp in the faster migrating form was higher for the C-terminal epitope-tagged forms of Dlp, presumably reflecting increased sensitivity to processing by Notum. The tag was inserted one residue from the GPI addition site.

(C) Lanes 1–4: immunoblot of S2 cells transfected to express Dlp-HA with or without Notum and treated with PI-PLC (probed with anti-HA). The slower migrating form was converted to the faster migrating form by PI-PLC, suggesting that the mobility difference is due to the GPI anchor.

(D) Immunoblot of S2 cells transfected to express Dlp-HA with or without Notum and subject to Triton X114 phase separation. Abbreviations: inp, input; det, detergent phase; aq, aqueous phase. Note that retention of the processed form in the detergent phase was considerably higher when unprocessed Dlp was present than when there was little unprocessed Dlp, perhaps suggesting interaction between the different forms.

(E) Immunoblot of S2 cells transfected to express GFP-Dlp, the truncated form GFP-Dlp-S987Z, or the S988P mutated form GFP-Dlp-S988P with wild-type or mutant versions of Notum (probed with anti-GFP).

(F) Immunoblots of S2 cells transfected to express GFP-Dlp-CD2 and probed with anti-GFP and anti-CD2. Lanes 1 and 2 show digestion with PI-PLC. Lanes 3–6 show the effects of cotransfection with wild-type and mutant Notum. As a control, lysates of cells transfected with GFP-Dlp-HA-C were digested in parallel with PI-PLC or cotransfected with notum (wt or mut). The results were identical to those in (B) (not shown).

4B). We noted that the C-terminally HA-tagged forms of Dlp were processed more efficiently than Dlp-HA, so that more of the faster migrating from was observed without addition of wild-type Notum (Notum is expressed in S2 cells). These observations are difficult to reconcile with Notum acting as a protease.

We next considered the possibility that the shift in Dlp mobility could be due to cleavage of the GPI anchor. Lysates were prepared from S2 cells transfected with Dlp-HA with or without Notum, and half of each was treated with phospholipase-C (PI-PLC) to cleave the GPI anchor of Dlp. Interestingly, PI-PLC cleavage caused a mobility shift in Dlp comparable to that caused by Notum (Figure 4C, lanes 2 and 3). Notum had no additional effect on PI-PLC-treated Dlp (lane 4). Although the magnitude of this shift in apparent molecular weight is larger than would be expected from the mass of the GPI anchor alone, anomalous migration of proteins in SDS-PAGE has been observed following removal of GPI anchors (Metz et al., 1994; Chen et al., 1998).

To further evaluate the possibility that the mobility shift caused by Notum might be due to cleavage of the GPI anchor, we performed a Triton X-114 phase separation analysis. Following phase separation, integral membrane proteins and GPI-anchored proteins are recovered mainly in the detergent phase, while other proteins are mainly in the aqueous phase. In cells cotransfected to express Dlp and Notum, processing of Dlp was efficient and the faster migrating form of Dlp was mainly recovered in the aqueous phase (Figure 4D). In cells transfected to express Dlp, the slower migrating form was mainly recovered in the detergent phase, consistent with it having a GPI anchor. The faster migrating form partitioned between aqueous and detergent phases. These observations are consistent with the suggestion that the faster migrating form of Dlp produced by Notum lacks a GPI anchor.

We next asked if Notum could act on forms of Dlp that lack a GPI anchor. The predicted GPI anchor site of Dlp consists of the sequence SDA (at S733) followed by a characteristic hydrophobic motif. Mutation of any of the three residues of the GPI anchor site to proline has been shown to reduce GPI addition in other glypicans (Udenfriend and Kodukula, 1995). The SDA motif was mutated to PDA in GFP-Dlp-S988P (residue numbers are for GFP-Dlp). We also prepared a truncated version of GFP-Dlp in which a stop codon was inserted after S987 to mimic the effects of cleavage of the protein chain at this position without addition of GPI. Coexpression with Notum had little or no effect on these forms of GFP-Dlp (Figure 4E). GFP-Dlp-S988P migrated predominantly at an intermediate position, faster than the

GPI-anchored form but slower than the Notum-processed form. This presumably reflects the full-length protein that has not been cleaved for GPI addition (this form appears as a minor band in most preparations). The major band of GFP-Dlp-S988P is unaffected by Notum, though a small amount of the protein does appear to have had GPI added (perhaps at S987) and was subject to Notum activity. The truncated GFP-Dlp-S987Z protein did not produce any of the slower migrating GPI anchored form and was not affected by Notum. GFP-Dlp-S987Z migrated slightly faster than the Notum-modified form of GFP-Dlp (Figure 4E). In Triton X-114 phase separation analysis, GFP-Dlp-S987Z partitioned into the aqueous phase (not shown), also consistent with the lack of a GPI anchor. We note that the form of Dlp cleaved by PI-PLC comigrated more closely with the Notum-processed form, consistent with the possibility that Notum cleavage was in the GPI moiety.

We next prepared a version of Dlp that is not GPI anchored to ask if it would be a substrate for Notum. GFP-Dlp was fused at residue S975 to residue R23 of the transmembrane protein CD2 and expressed in S2 cells. This protein contains the entire ectodomain (CRD and stem) of Dlp, but lacks the GPI addition signals (the fusion is at the equivalent position in Dlp to the S987Z construct; numbering differs because the HA tag was removed). GFP-Dlp-CD2 was not cleaved by PI-PLC (Figure 4F, lanes 1 and 2), verifying that it does not contain a GPI anchor. GFP-Dlp-CD2 was also not a substrate for Notum (lanes 3–6). If Notum acted as a protease to cleave in the Dlp protein, we would have expected GFP-Dlp-CD2 fusion to be a substrate for Notum. Likewise, if Notum acted on the GAG side chains or on another posttranslational modification, we would have expected GFP-Dlp-CD2 to be affected. Taken together, these observations indicate that Notum can induce modification of Dlp in a manner that resembles cleavage of the GPI anchor. Given that the α/β-hydrolases includes lipases, it is possible that Notum might act directly to cleave within the GPI anchor. Alternatively, Notum could induce the activity of an endogenous phospholipase.

Notum-Processed Dlp Is Shed from Cells
Can cleavage at the GPI anchor cause Dlp to be shed from the cell surface? The faster and slower migrating forms of Dlp-HA were efficiently recovered from the cell lysates (Figure 5A). In addition, the faster migrating cleaved form of Dlp-HA was recovered from the culture medium of cells overexpressing Notum. The slower migrating GPI-anchored form of Dlp-HA was not recovered from the medium. Very little Dlp-HA was recovered from medium conditioned by cells transfected with Dlp alone or with the Notum mutant. This indicates that Notum can release Dlp from the cell surface, causing it to be shed into the medium. Notum did not induce detectable shedding of HA-tagged Dally.

We next expressed Dlp-HA or Dally-HA under en-Gal4 control in the wing disc to compare the effects of the endogenous Notum protein on Dlp and Dally. Notum is expressed at the dorsoventral boundary under control of Wg. En-Gal4 produced a uniform level of Dally-HA across the wing pouch (Figure 5C). In contrast, Dlp-HA protein levels were lower at the dorsoventral boundary
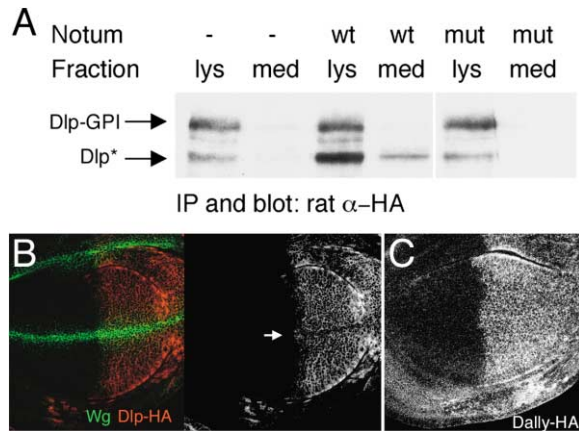

Figure 5. Notum-Induced Shedding of Dlp
(A) Immunoblot of Dlp-HA from S2 cell lysates and conditioned medium fractions from cells transfected with Dlp-HA alone, or together with wild-type Notum (wt) or the S237A Notum mutant (mut). The medium fractions were immunoprecipitated with rat anti-HA. Secreted Dlp-HA was detected in significant amounts only when the S2 cells were cotransfected with Dlp-HA and Notum-wt.
(B) Wing disc expressing UAS-Dlp-HA under control of en-Gal4. Dlp-HA is shown in red and separately at right. The arrow indicates reduced Dlp-HA expression at the DV boundary, where Wg (green) is expressed.
(C) Wing disc expressing UAS-Dally-HA under control of en-Gal4. Dally-HA was not decreased at the DV boundary.

(Figure 5B). Notum caused loss of Dlp, but had little effect on Dally. This presumably reflects shedding of Dlp protein from cells in the disc in the region where Wg levels are highest.

Genetic Interaction between Notum and Dlp
The finding that Notum can cleave and release Dlp from cells raised the possibility that Dlp might be released together with bound Wg. This could explain how Dlp acts to reduce Wg activity where Wg and Notum levels are high. We therefore tested the possibility of synergistic action of Notum and Dlp on Wg function. Overexpressed Dlp binds and sequesters Wg at the cell surface, leading to reduced Wg activity (Baeg et al., 2001). Coexpression of Notum with Dlp reduced the ability of Dlp to bind and retain Wg (Giraldez et al., 2002). To examine the effects of Notum on Dlp activity, we selected transgenes expressing Notum or Dlp at levels that were on the threshold for altering wing morphology. When expressed under en-Gal4 control, each transgene caused loss of some of the posterior wing margin bristles, indicating mild reduction of Wg activity (Figures 6A and 6B). Expression of the two transgenes together caused scalloping of the wing (Figure 6C), a typical defect caused by more severe reduction of Wg activity. This observation suggests that expression of Notum enhanced the ability of Dlp to reduce Wg activity. In view of the finding that Notum can cause Dlp to be released from the cell surface, we suggest that coexpression of Notum causes Dlp to be released together with bound Wg (Notum reduced the level of Wg bound in the disc; Giraldez et al., 2002). Release of Wg bound to Dlp could reduce the level of Wg available for signaling and cause
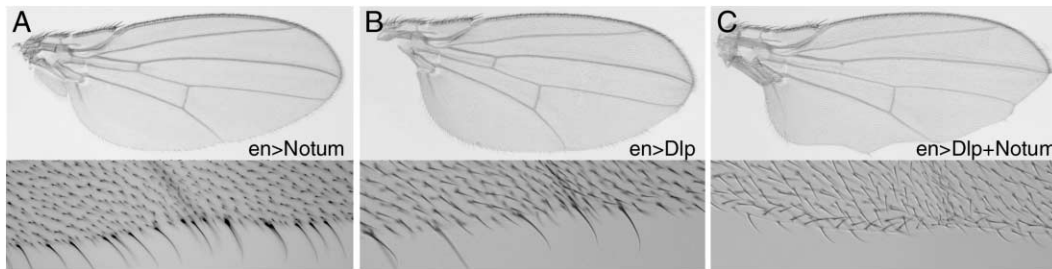
Figure 6. Synergistic Interaction of Notum and Dlp

(A and B) Cuticle preparations of adult wings of the following genotypes: (A) en-Gal4 UAS-Notum, (B) en-Gal4 UAS-Dlp-HA. Note the slightly reduced number of posterior margin bristles (see lower panels).
(C) en-Gal4 UAS-Notum + UAS-Dlp-HA wing. Note the scalloping of the wing in the posterior compartment.

the observed wing scalloping phenotype. Notum causes release of Dlp from cells at the DV boundary, where Wg levels are highest.

Discussion

One surprising outcome of this study is the finding that reduced Dlp activity has opposing effects on the Wg activity gradient at low and high levels of Wg (see also Kirkpatrick et al., 2004 [this issue of *Developmental Cell*]). Previous studies have shown that Dlp can bind Wg at the cell surface and suggested that Dlp might serve as a coreceptor to facilitate binding of Wg to the D-fz2/Arrow receptor complex (Baeg et al., 2001). Our findings show that reduced Dlp levels cause a reduction in Wg signaling activity in part of the wing disc. While this is compatible with a coreceptor model, we note that overexpression of Dlp leads to a net reduction in signaling even though cell surface Wg levels are elevated (Baeg et al., 2001). We prefer the alterative explanation that Dlp provides a large number of low-affinity binding sites that help to retain Wg near the cell surface so that Wg has a chance to interact with its receptor in regions of the disc where its levels would otherwise be too low. This would not require a direct involvement of Dlp in mediating Wg-D-Fz2 interaction, but does not exclude it. Wg can signal in the absence of Dlp in a cell culture assay, indicating that it is not an essential cofactor (Lum et al., 2003).

We have presented evidence that Notum can induce cleavage of Dlp in a manner that resembles PI-PLC cleavage of the GPI anchor. Notum and PI-PLC produce comparable shifts in the electrophoretic mobility of Dlp. Although the magnitude of this shift is larger than would be expected from the mass of the GPI anchor, other studies have shown that the effects of removing GPI anchors are not predictable. Examples of increased or decreased mobility have been reported, and the magnitude of the shifts can be large (e.g., Metz et al., 1994; Chen et al., 1998). Notum-induced processing also renders Dlp soluble in the aqueous phase following detergent phase separation of soluble and membrane-associated proteins, consistent with removal of the GPI anchor. Third, a Dlp-CD2 fusion protein that is not GPI anchored, and is therefore not a substrate for PI-PLC, is insensitive to Notum. This would not be expected if Notum acted on the GAG side chains or if Notum was

a protease cutting within the Dlp core protein. These observations are consistent with two modes of Notum action. Notum might act directly to cleave the GPI anchor or it might modify Dlp in some way that makes Dlp a substrate for an endogenous phospholipase. The exact nature of the Notum-induced cleavage remains to be determined; however, one intriguing possibility is that Notum might cleave within the glycan linker of the GPI anchor.

Can the effects of Notum on Dlp provide an explanation for the apparently opposing activities of Dlp at high and low levels of Wg? How could Notum-induced cleavage of Dlp lead to reduced Wg activity, whereas removal of Dlp by RNAi increases Wg activity? If Wg remains bound to Dlp when Dlp is cleaved and shed from the cell, bound Wg would also be shed and so become unavailable for signaling (see Figure 7). Shedding of Dlp as a consequence of Notum-induced cleavage could reduce peak levels of available Wg. Notum is expressed at the source of Wg and so would be expected to shed Dlp and reduce Wg activity where Wg levels are highest. In this way, removal of Dlp protein as a consequence of Notum-induced cleavage could have a different effect than failure to express Dlp. In the absence of Dlp, Wg would not bind Dlp and be shed with it, so that more Wg might be available to interact with Dally and/or the Wg receptor complex. This could increase the effective concentration of Wg locally near the site of Wg production.

This model is consistent with the observed synergy between low-level expression of Dlp and Notum. Overexpression of Dlp is thought to increase the number of Wg binding sites and shift the equilibrium toward more Wg bound to Dlp. At high levels of Dlp, this can reduce the amount of Wg available for signaling and produce a Wg loss-of-function phenotype (Baeg et al., 2001). We have chosen a level of Dlp overexpression that produces a mild defect due to reduced availability of Wg. Coexpression of Notum would lead to shedding of the Dlp bound Wg and thus remove this fraction of Wg from the pool on the cell surface so that it would no longer be able to contribute to the pool of Wg in equilibrium with the receptor. This would be expected to further reduce Wg activity and increase the severity of the defect, as observed.

In a recent report, Han et al. (2004) presented evidence that the two glypicans Dally and Dlp function redundantly in the wing disc in Hh signaling. They suggest a
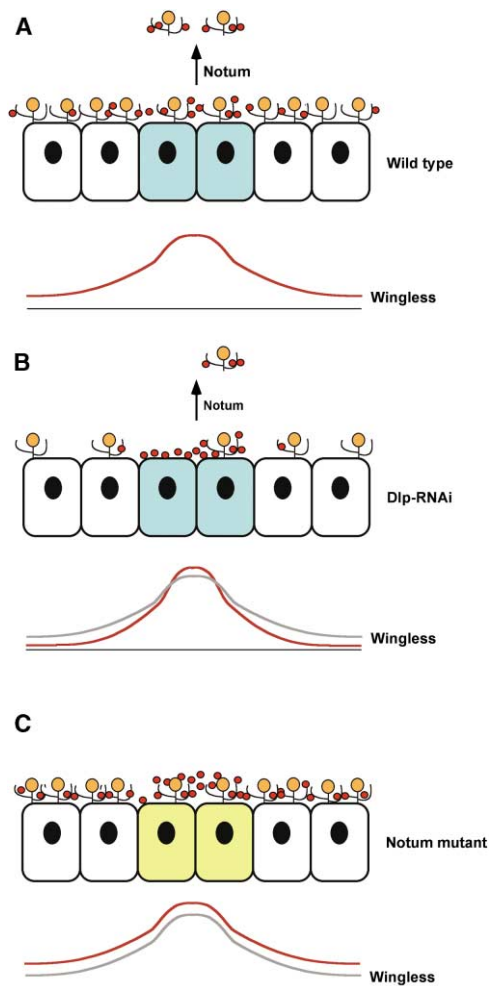
Figure 7. Model for the Effect of Notum on Dlp and Wingless Distribution

(A) Schematic representation of a wild-type wing imaginal disc. Cells at the DV boundary (marked with blue) produce Wg (red) and Notum. Notum induces the release of Dlp (yellow) from its GPI anchor. This localized shedding of Dlp, together with bound Wg, shapes the Wg gradient (red line, shown below).
(B) Less Wg is shed as a result of Dlp depletion. This results in Wg accumulation and a mild Wg gain-of-function phenotype. However, lack of GPI-anchored Dlp in regions of the disc where Notum is not expressed (and Dlp not shed) impairs Wg retention and formation of the Wg gradient (red line, wild-type Wg distribution gray line).
(C) In the absence of Notum (yellow), Dlp is not shed and Wg accumulates at the DV boundary and subsequently throughout the wing disc.

role for glycans in ligand movement. In this context, it is interesting that Dally hypomorphic mutants show a weak Wg loss-of-function phenotype at the wing margin (Fujise et al., 2001), the opposite of what we find for Dlp by RNAi and that Kirkpatrick et al. (2004) find for *dlp* mutants. Our findings suggest that the increase in Wg activity near the wing margin may be a consequence of the relationship between Dlp and Notum. Notum does not appear to cleave Dally efficiently, and so it may be that Dally mutants can only reduce Wg activity. We speculate that Dally and Dlp are likely to be redundant in Wg signaling in regions of lower Wg activity, outside the domain of Notum activity.

Localized cleavage of Dlp induced by regulated expression of Notum may provide a unifying explanation for the opposing effects of the Dlp glypican in different regions of the tissue. We note that a recent mathematical model of Wg gradient formation invoked a need for an elevated level of Wg turnover in cells close to the source of Wg (Eldar et al., 2003). Although the mechanism described here differs from that suggested by Eldar, it would be sufficient to provide the reduction of Wg activity in cells closest to the source of the secreted ligand that is needed for formation of a robust morphogen gradient.

Experimental Procedures

Drosophila Strains
sd-Gal4, ap-Gal4, and en-Gal4 are described in flybase. UAS-Notum, UAS-Notum S237A, UAS-Dlp-HA(wt), and UAS-Dally-HA(wt) are described in Giraldez et al. (2002).

Sources of Antibodies
Rabbit anti-Vestigial (Williams et al., 1993), mouse anti-Wg (Brook and Cohen, 1996), mouse anti-Senseless (Nolo et al., 2000), rat anti-HA (Roche), rabbit anti-GFP (Torrey Pines Laboratories), and mouse anti-ratCD2 (Serotech) were used. Rat anti-Dll was produced by Jun Wu. Anti-Dlp was raised in rats to a fragment comprising aa 44–730 of Dlp.

Constructs
The insert for UAS-Dlp-RNAi construct was amplified by PCR with EcoRI sites added and cloned into SympUAST (Giordano et al., 2002). UAS-Dlp(-HS) was prepared by deleting aa 624–646. A fragment from aa 647 to the Stop codon was amplified and used to replace the NdeI-EcoRI 3′ fragment of pKS-Dlp (Baeg et al., 2001). The HA tag was introduced at the NdeI site. Finally, the SG site at aa #670 was converted to AG by PCR. The modified Dlp cDNA was cloned in pUAST. Dally-HA cDNA was amplified from *hs-HA-dally* (Tsuda et al., 1999) and cloned into pUAST. UAS-Dally(-HS) was prepared by conversion of SG sites at aa 549, 569, 573, 597, and 601 into AG by PCR. Dlp-HA-C was prepared by introducing an Nhe site and the HA tag at S732 (inserted sequence: ASYPYDVPDYAAS). GFP-Dlp-S987Z and GFP-Dlp-S988P were prepared by PCR to change the codon for S987 from TCA to TAA and for S988 from TCC to CCC. GFP-Dlp-CD2 was produced by replacing the C terminus of GFP-Dlp-HA-C from the Nhe site at S975 with a fragment of rat CD2 beginning at residue R23 (after the signal peptide). Details available on request.

Ion-Exchange Chromatography
Cells were lysed in 10 mM Tris (pH 7.4), 0.14 M NaCl with protease inhibitors. 50 μl was added to 450 μl of 10 mM NaPO$_4$ (pH 6) containing 0, 0.14, 0.3, 0.5, or 1.0 M NaCl (with protease inhibitors), mixed with 50 μl Q-Sepharose resin (Amersham) pre-equilibrated in the same buffers, and incubated at 4°C for 1 hr. The resin was washed with 10 vol of the respective buffer and bound protein was released with 50 μl 3 M NaCl.

PI-PLC Treatment
10 μl of S2 cell lysate was incubated for 3 hr at 37°C with 50 mU PI-PLC (Sigma) in 0.2% Triton X-100, 70 mM NaCl, 10 mM EDTA, 2.5 mM Tris-HCl (pH 8), with protease inhibitors (total vol. 50 μl).

Heparitinase Treatment
Heparitinsae I and 3G10 antibody were obtained from Seikagaku (USA). 20 μl samples of S2 cell lysates were digested according to manufacturer's instructions.

Dlp Secretion Assay
S2 cells were transfected with 1–2 μg of pMT-Gal4 and UAS-Dlp and UAS-Notum or UAS-Notum S237A (active site mutant) or empty

vector using 4 μl of CellFectin (Invitrogen) per well. Cells were recovered for 6 hr after transfection, induced for 2 days with 0.7 mM CuSO₄, and lysed in 150 μl of 5 mM Tris, 150 mM NaCl, 1% Triton X-100 (pH 8). 1/10th was analyzed on SDS-PAGE. Medium fractions (1 ml) were immunoprecipitated with 1 μg anti-HA overnight at 4°C. 100 μl of protein G slurry was added for 30 min at 4°C. The beads were washed three times with PBS, 0.1% Triton X-100, and then boiled in 50 μl 2 × SDS-PAGE loading buffer and 1/3 loaded on SDS-PAGE. All buffers were supplemented with protease inhibitors (Boehringer).

Phase Separation
Triton X-114 phase separation of total S2 cell lysates from cells transiently transfected to express Dlp-HA, Dlp-HA with Notum, or GFP-Dlp-S987Z was performed as described by Bordier (1981).

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