Glypicans shunt the Wingless signal between local signalling and further transport

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Summary

The two glypicans Dally and Dally-like have been implicated in modulating the activity of Wingless, a member of the Wnt family of secreted glycoprotein. So far, the lack of null mutants has prevented a rigorous assessment of their roles. We have created a small deletion in the two loci. Our analysis of single and double mutant embryos suggests that both glypicans participate in normal Wingless function, although embryos lacking maternal and zygotic activity of both genes are still capable of transducing the signal from overexpressed Wingless. Genetic analysis of *dally-like* in wing imaginal discs leads us to a model whereby, at the surface of any given cell of the epithelium, Dally-like captures Wingless but instead of presenting it to signalling receptors expressed in this cell, it passes it on to neighbouring cells, either for paracrine signalling or for further transport. In the absence of *dally-like*, short-range signalling is increased at the expense of long-range signalling (reported by the expression of the target gene *distalless*) while the reverse is caused by Dally-like overexpression. Thus, Dally-like act as a gatekeeper, ensuring the sharing of Wingless among cells along the dorsoventral axis. Our analysis suggests that the other glypican, Dally, could act as a classical co-receptor.

Key words: Wingless, Glypicans, Morphogen transport, *Drosophila*, Signalling

Introduction

Extracellular ligands are typically degraded after engagement with their cognate receptors and activation of the signalling pathway. Degradation is usually initiated by receptor-mediated endocytosis. This raises a fundamental problem for ligands that must act over long distances within epithelia. High-affinity signalling receptors are expected to trap the ligand near the source and thus prevent transport to more distant cells (Chen and Struhl, 1996; Kerszberg and Wolpert, 1998). How is a locally produced ligand appropriately allocated to all the cells within a field, near and far? One possibility is that specialised, non-signalling receptors ensure long-range signalling. In wing imaginal discs of *Drosophila*, the secreted glycoprotein encoded by *wingless* is produced at the prospective wing margin and spread symmetrically along the dorsoventral axis (Zecca et al., 1996; Cadigan et al., 1998). One class of receptors that could modulate the distribution of Wingless are heparan sulphate proteoglycans (HSPGs). In particular, overexpression of the glypican Dally-like (Dlp) causes accumulation of Wingless at the surface of imaginal disc cells (Baeg et al., 2001). Somehow, such accumulation is accompanied by a reduction in Wingless signalling. Although this gain-of-function experiment suggests that Dally-like could regulate the distribution of Wingless, so far, this has not been confirmed by loss-of-function analysis. Indeed, the normal role of Dlp in Wingless function is still unknown.

Good evidence for the involvement of HSPGs in Wingless function comes from the isolation and analysis of mutants in genes encoding HSPG modifying enzymes. *Drosophila* embryos lacking *sugarless*, which encodes UDP-Glucose-Dehydrogenase, an enzyme required for heparan sulphate biosynthesis, are deficient in Wingless signalling (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997). Likewise, embryos lacking *sulfateless*, which encodes heparan sulphate N-deacetylase/N-sulphotransferase (an enzyme needed for the modification of heparan sulphate) also resemble *wingless* mutants (Lin and Perrimon, 1999). Further genetic analysis showed that HSPG metabolising enzymes are also implicated in additional signalling pathways such as those activated by Hedgehog, FGF and Dpp (Bellaiche et al., 1998; Lin et al., 1999; Han et al., 2004a; Takei et al., 2004). To what extent HSPGs play distinct roles in the different signalling pathways remains to be determined. Practically, the involvement of HSPGs in both Wingless and Hedgehog signalling complicates genetic analysis in embryos because the terminal phenotype of *wingless* and *hedgehog* mutants look very similar to each other, making it difficult to distinguish roles in either or both signalling pathways. In imaginal discs, Wingless and Hedgehog signalling are easily distinguished experimentally and, in this tissue, it is clear that loss of *sulfateless* causes a reduction in Wingless signalling (Baeg et al., 2001). In particular, Wingless protein that normally accumulates at the surface of wild type Wingless-expressing cells does not do so at the surface of *sulfateless* mutant cells, suggesting a potential role of HSPGs in tethering Wingless at the cell surface.

Genetic analysis of HSPG modifying enzymes clearly

implicates HSPGs in Wingless function. What are the relevant HSPGs involved? Four HSPGs are predicted to be encoded by the *Drosophila* genome (Nybakken and Perrimon, 2002). Of these, as mentioned above, Dlp is a good candidate on the basis of gain-of-function studies. However, RNAi injection in embryos has failed to confirm a role for Dlp in Wingless signalling. Instead, a clear role in Hedgehog signalling was demonstrated by RNAi (Desbordes and Sanson, 2003; Lum et al., 2003) and this was later confirmed with traditional genetic mutants (Han et al., 2004b). In these experiments, a possible role of Dally-like in Wingless signalling might have been masked by redundant activity from another glypican. Indeed, the other glypican found in Drosophila, Dally, has been implicated in Wingless function on the basis of RNAi-induced phenotypes in embryos (Lin and Perrimon, 1999) although this has been questioned by subsequent experiments (Desbordes and Sanson, 2003). Nevertheless, weak *wingless*-like phenotypes are seen in presumed weak allele of *dally* (Lin and Perrimon, 1999; Tsuda et al., 1999).

Overall, various experiments involving presumed weak alleles, RNAi injection or overexpression have implicated Dally and Dlp in Wingless function (Nakato et al., 1995; Lin and Perrimon, 1999; Tsuda et al., 1999; Baeg et al., 2001). However, a clear assessment of their role has been hampered by the involvement of HSPGs in other signalling pathways and also by the lack of suitable null mutants (Sanson, 2004). We have created null mutants in both *dally* and *dlp*. The analysis of single and double mutant embryos shows that both glypicans participate in normal Wingless function. Using a combination of gain- and loss-of-function approaches in imaginal discs, we have investigated the specific roles of the two glypicans in regulating the activity and distribution of the Wingless signal in wing imaginal discs.

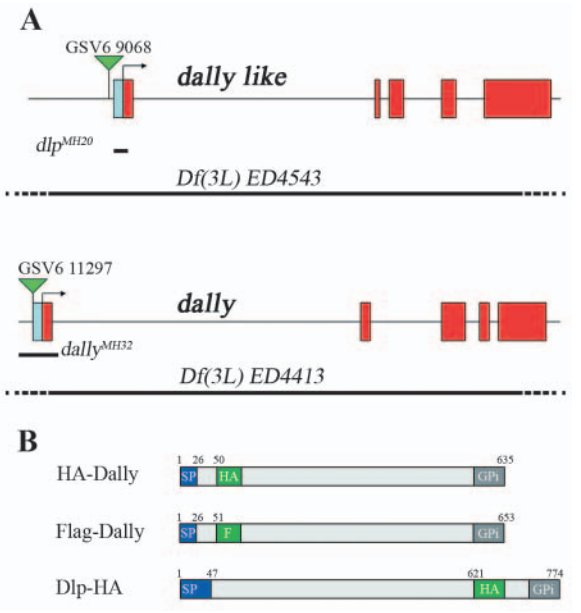

Fig. 1. Molecular lesions in dlp^{MH20} and $dally^{MH32}$ and organisation of the tagged glypicans used. (A) Diagram of the genomic regions of *dlp* and *dally*. The position of the original P-elements is shown with an inverted triangle. The deletions in dlp^{MH20} and $dally^{MH32}$ are indicated as small black bars (to scale). The exact break points relative to the predicted start of transcription are –42 to +467 for *dlp* (ATG is at 454) and –472 to +1410 for *dally* (ATG is at 728). The deficiencies used in complementation assays uncover the whole region with break points located far away and are represented as long black lines [*Df(3L) ED4413 dally* was generated for this study while *Df(3L) ED4543 dlp* was obtained from Drosdel]. (B) The tagged glypicans used in this study. The location and nature of the tag is shown. HA-Dally was only expressed in cultured cells while Flag-Dally and Dlp-HA were expressed in transgenic flies and cells.

Materials and methods

DNA constructs

To create an N-terminal FLAG tagged version of Dally, a double-stranded oligonucleotide containing three copies of the FLAG epitope was inserted in the unique *Aat*II site (position 150 in Dally). The Dally-FLAG cDNA was subsequently inserted in pUAST as an *Eco*RI-*Xba*I fragment. DNA carrying UAS-Dally-HA and UAS-Dlp-HA as well as pMT-Gal4 used for the binding experiments with S2 cells were obtained from S. Cohen, EMBL. See Fig. 1B for a diagram of the constructs.

Drosophila stocks

The following transgenic stocks were used for exogenous expression: UAS-Dally-FLAG (generated for this study), UAS-Dally-like-HA (from S. Cohen, EMBL). Both were driven with *dpp-Gal4*, *engrailed-Gal4* or *tubulin-Gal4*. For loss-of-function studies, we generated mutants in *dally* and *dlp* by imprecise excision of a P-element inserted in the 5′ region (see Fig. 1A). For *dlp*, we obtained a deletion of nearly 500 nucleotides, which removes the 5′UTR and 16 nucleotides downstream of the ATG. For *dally,* we obtained a deletion of about 1.8 kb that removes the first exon. Both mutants are expected to produce no functional protein. Clones of mutant cells were induced by Flp-mediated mitotic recombination. The following additional stocks were used: GSV6 9608 and GSV6 11297 (P-insertions in *dlp* and *dally*, respectively, obtained from Toshiro Aigaki, Tokyo University); *Df(3L) ED4413*, a large deficiency that uncovers *dally* (generated by us with the Drosdel kit, www.drosdel.org.uk); and *Df(3L) ED4543* (obtained from Drosdel), which uncovers *dlp*. *w*; FRT2A dlp^{MH20}, *w*; FRT2A $dally^{MH32}$ and *w*; FRT2A $dally^{MH32}dlp^{MH20}$ were generated for this study. We also used *yw* hsflp; FRT2A *ubi-GFP* (from the Bloomington Stock Center), wg^{CX4} and hh^{AC} (described at flybase.bio.indiana.edu), UAS-GFP-Wingless, and *sim-gal4* (from Benny Shilo, Weizmann Institute). Germline clones were induced with *yw*; FRT2A ovo^{D} (from the Bloomington Stock Center). To induce overexpression clones, the following stocks were used: *yw* hsflp; *tubulin>>GAL4>>UAS lacZ, yw; FRT42D pwn* and *yw hsflp; FRT42D Gal-80; tubulin-Gal-4* (from S. Cohen, EMBL).

Embryo preparations

To visualise cuticle patterns, 24-hour-old embryos were mounted in Hoyer's and photographed under DIC or phase-contrast microscopy. In situ hybridisation was performed on fixed embryos hybridised with a digoxigenin labelled *rhomboid* probe (a gift from J. F. de Celis, Cambridge University).

Antibodies

Primary antibodies used were mouse anti-Wingless 4D4 (prepared from cells obtained from the DSHB), mouse M2 anti-Flag (Sigma; 1/1000), mouse anti-HA 1.1 (Covance; 1/500), Alexa-488 labelled mouse anti-HA 1.1 (Covance; 1/500), rabbit anti-GFP (Abcam; 1/2500), mouse anti Distal-less (from I. Duncan, University of Wisconsin; 1/500) and guinea pig anti-Senseless (from Hugo Bellen, Baylor College of Medicine; 1/1000). Secondary antibodies (all from Molecular Probes) were Alexa488-conjugated goat anti-rabbit (1/200), Alexa488-conjugated goat anti-mouse (1/200), Alexa594-

conjugated goat anti-mouse (1/200) and Alexa488-conjugated goat anti-guinea pig (1/200).

Wingless-binding assay

GFP-Wingless medium prepared from a stable line (S2-GFP-Wg) was applied to S2 cells grown on coverslips and transiently transfected with *dally* or *dlp* constructs (HA-tagged) and induced overnight with 0.25 mM $CuSO_4$. GFP-Wingless binding was performed on ice as previously described (Bhanot et al., 1996). After fixation, GFP-Wingless was detected with an anti-GFP antibody and the HA tagged receptors with anti-HA.

Results and discussion

Dally and Dlp contribute to normal Wingless activity in embryos

In order to assess the function of Dally and Dlp, we have created, for each gene, a small deletion that removes the translation start and the signal peptide. These alleles are henceforth referred to as $dally^{MH32}$ and dlp^{MH20} (see molecular description in Fig. 1). For both alleles, even if an opportunistic translation start were used, no signal peptide would be encoded. For *dlp*, the next possible ATG is 123 residues downstream of the normal translation start, while for *dally* the mutation removes the first exon thus deleting at least the first 90 residues. Therefore, in both cases, any protein product would not be allowed to enter the secretory pathway and reach the cell surface even if a downstream translation start is used (i.e. no functional protein could be produced). On the basis of this evidence, we consider dlp^{MH20} and $dally^{MH32}$ to be null. Previous *dally* alleles, including the strongest one, $dally^{P2}$ were thought to be hypomorphic. However, we find that the penetrance and expressivity of adult phenotypes is similar in $dally^{MH32}$ homozygotes as in $dally^{P2}$ homozygotes (as well as in $dally^{MH32}$/*Df(3L) ED4413* animals). It appears therefore that $dally^{P2}$ is also a null allele.

As a first assessment of the role of these two proteoglycans in Wingless function, we analysed embryos that lack the maternal and zygotic contribution of either *dally*, *dlp*, or both. Embryos lacking *dally* appear normal, suggesting that this gene is not essential for embryonic development (data not shown) (see also Desbordes and Sanson, 2003). By contrast, and as previously shown, Dlp-deficient embryos die with a so-called denticle lawn phenotype, which has been attributed to a failure in transduction of the Hedgehog signal [early targets of Hedgehog signalling fail to be activated (Desbordes and Sanson, 2003; Lum et al., 2003)]. Assessing a possible, additional role in Wingless signalling has been difficult because the terminal phenotype of embryos lacking Hedgehog signalling is similar to that of *wingless*-deficient embryos. To investigate this, we used an epidermal molecular marker that differentiates between *hedgehog*, *wingless* and *wingless hedgehog* mutant embryos. As can be seen in Fig. 2A-C, the pattern of *rhomboid* expression is distinct in all three genetic conditions (see also Alexandre et al., 1999). In embryos lacking maternal and zygotic *dlp*, the pattern of *rhomboid* expression is variable with features of both *hedgehog* and *wingless* mutants, but falling short of phenocopying *wingless hedgehog* double mutants (not shown). This suggests a loss of Wingless activity, although an incomplete one, perhaps because of a redundant

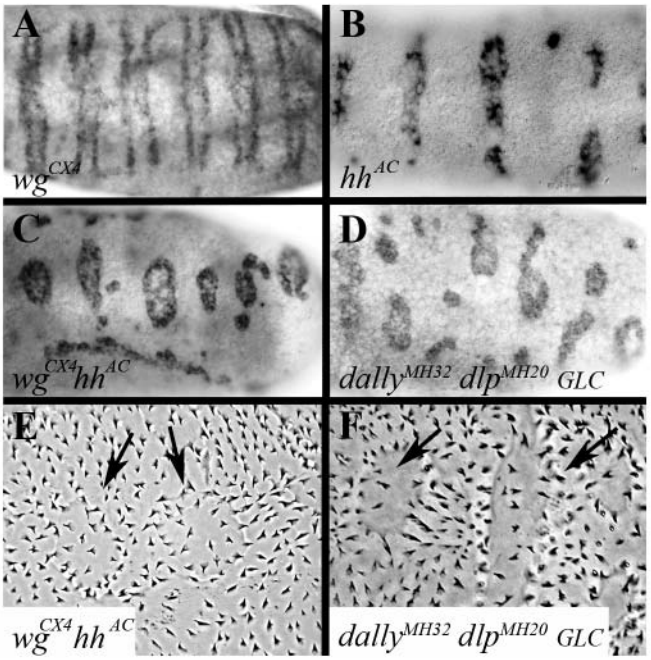

Fig. 2. Glypicans are required for Wingless activity in embryos. (A-D) Expression of *rhomboid* in various mutant embryos at about stage 13. (A) Expression of *rhomboid* in *wingless* mutants is characterised by a 'tramtrack pattern' (this is seen in all embryos, *n*=100). (B) No such pattern is seen in *hedgehog* mutants, which express *rhomboid* in broadened stripes compared with wild type (stripes become occasionally split as shown on the right hand side). (C) In *wingless hedgehog* mutants, *rhomboid* stripes collapse into ventral rings (this is true for most segments in all embryos, *n*=53). Such rings are never seen in *wingless* mutants, *n*=100, and rarely so in hedgehog mutants (12% of *hedgehog* mutants have one or two ventral rings, none have more *n*=25). (D) The phenotype of embryos lacking maternal and zygotic *dally* and *dlp* is similar to – though slightly more variable than – that of *wingless hedgehog* mutants (a majority of abdominal *rhomboid* stripes collapse into rings in 69% of embryos and all embryos have at least one collapsed stripe, *n*=26). (E) Cuticle phenotype of *wingless hedgehog* double mutant. The epidermis is covered with denticles (the lawn phenotype) and two mid-ventral 'whorls' can be seen (arrows). Whorls are sometimes seen in *hedgehog* mutants but not in the mid-ventral region. (F) The phenotype of embryos lacking maternal and zygotic *dally* and *dlp* is similar although more variable with fewer less marked mid-ventral whorls. This suggests that weak residual signalling could occur in the absence of the glypicans.

contribution from Dally. We therefore assessed the pattern of *rhomboid* expression in embryos lacking maternal and zygotic *dally* and *dlp* (*dally dlp* double mutants for short). As shown in Fig. 2D, it strongly resembles that seen in *wingless hedgehog* double mutants. This similarity is confirmed by close inspection of terminal cuticular phenotypes. The cuticle phenotype of *dally dlp* double mutants is more severe than that of embryos lacking maternal and zygotic *dlp* (not shown) and, importantly, it is characterised by a feature seen in *wingless hedgehog* double mutants, the presence of mid-ventral denticles whorls (Fig. 2E,F). We conclude that both glypicans participate in the normal activity of Wingless and Hedgehog.

Because *dally dlp* double mutant embryos are deficient in

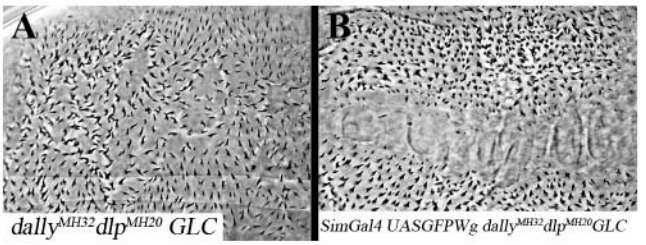

Fig. 3. Signal transduction in *dally dlp* mutants expressing exogenous Wingless. (A) Lawn phenotype of an embryo lacking maternal and zygotic *dally* and *dlp*, as shown in Fig. 2E. (B) Naked cuticle is induced along the ventral region by expression of GFP-Wingless under the control of sim-gal4 in *dally dlp*-deficient embryos [as used by Desbordes and Sanson (Desbordes and Sanson, 2003)]. This shows that signal transduction can take place in the absence of the glypicans if sufficient Wingless expression is sustained.

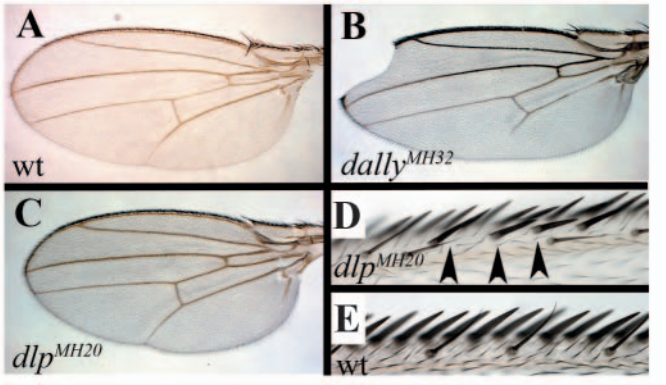

Fig. 4. Wing phenotypes in mutant flies. (A) Wild type. Homozygous *dally* mutant flies survive and sometimes (5%) display notches in the margin, which are symptomatic of reduced Wingless signalling (B). Distal truncation of vein 5 is also frequent but we have not attempted to characterise this further. The few homozygous *dlp* mutants that survive to adulthood (around 1%) have wings characterised by two fully penetrant phenotypes: a narrowing of the space between veins 3 and 4 (C), which suggests reduced *hedgehog* signalling (Crozatier et al., 2002) and (D) the formation of ectopic bristles on either side of the margin (arrowheads, compare with wild type shown in E), an indication of excess Wingless signalling. The same phenotypes are seen in surviving flies carrying the mutation (dlp^{MH20} and $dally^{MH32}$) over a large deficiency (*Df(3L) ED4543 dlp* and *Df(3L) ED4413 dally*, respectively).

Hedgehog signal transduction, they express only a small amount of *wingless* during a short period [*wingless* transcription is maintained by Hedgehog signalling (Lee et al., 1992)]. We therefore assayed the effect of artificially sustained Wingless expression in the absence of Dally and Dlp. As shown in Fig. 3, expression of *UAS-GFP-Wingless* under the control of *sim-gal4* leads to local activation of Wingless signalling in *dally dlp* double mutants, as evidenced by the formation of naked cuticle in the mid ventral region. Therefore, *dally* and *dlp* are not absolutely essential for Wingless signal transduction. Their roles in the embryo could be to boost or sustain the signal, perhaps by ensuring sufficient retention/accumulation of Wingless at cellular interfaces. In any case, the role of Dlp in Wingless function seems to predominate because embryonic Wingless signalling appears normal in the absence of Dally. However, Dally must provide some activity that contributes to normal Wingless function because the phenotype of *dally dlp* mutants is stronger than that of *dlp* single mutants.

At the prospective wing margin, *dlp* mutants display excess Wingless signalling while *dally* mutants suffer from a loss of Wingless signalling

To further investigate the roles of Dally and Dlp in Wingless function, we analysed the phenotypes of adult mutants. As mentioned above, embryos lacking all *dally* function are normal. In fact, they give rise to viable adults. These have recognisable phenotypes in a variety of tissues, as reported previously for presumed weaker alleles. This includes reduced and rough eyes, disrupted antennae, loss of bristles and, in most males (occasionally in females), loss of external genital structures (Nakato et al., 1995). We concentrate on the wing because it allows a relatively simple assessment of Wingless function. In wing imaginal discs, *dally* transcription is upregulated near the Wingless source (Nakato et al., 1995), suggesting a role at the prospective margin. As shown in Fig. 4B, $dally^{MH32}$ homozygotes display notches in the wing margin at a low frequency (5%) (see also Nakato et al., 1995). Interestingly, the frequency and severity of these notches is significantly higher (41%) in adults arising from maternal null embryos (in an otherwise identical genetic background), suggesting that the maternal contribution of *dally* is long lasting. Because loss of margin tissue is indicative of insufficient Wingless signalling (Phillips and Whittle, 1993), we conclude that wild type Dally contributes positively to Wingless function.

Contrary to a previous report (Han et al., 2004b), we find that *dlp* is also not zygotically required for adult viability: dlp^{MH20} homozygous flies (with wild-type maternal contribution) survive to adulthood, albeit at a low frequency. Therefore, the maternal contribution of *dlp* can support development to adulthood, thus enabling us to assess the phenotype in wings. Here, in situ hybridisation shows that *dlp* is uniformly expressed except at the Wingless source where is it downregulated (not shown). Surprisingly, dlp^{MH20} wings display a weak but fully penetrant phenotype that is characteristic of apparent excess Wingless signalling. During normal wing development, specialised bristles form at the prospective wing margin, where Wingless signalling is highest during imaginal disc development (Couso et al., 1994). In the wings of surviving dlp^{MH20} homozygotes, supernumerary bristles form on either side of the margin (Fig. 4D), an indication of ectopic Wingless signalling (Couso et al., 1994). Therefore, loss of *dlp* activity leads to excess Wingless signalling, at least around the wing margin. It is intriguing that mutations in *dally* and *dlp* lead to qualitatively different phenotypes with respect to Wingless signalling at the wing margin, while these two genes appear to be redundant for viability (homozygous double mutants die at the end of embryogenesis, even in the presence of maternal contribution). One possible explanation is that the two glypicans could perform distinct cell biological functions that together contribute to optimal Wingless activity (see below).

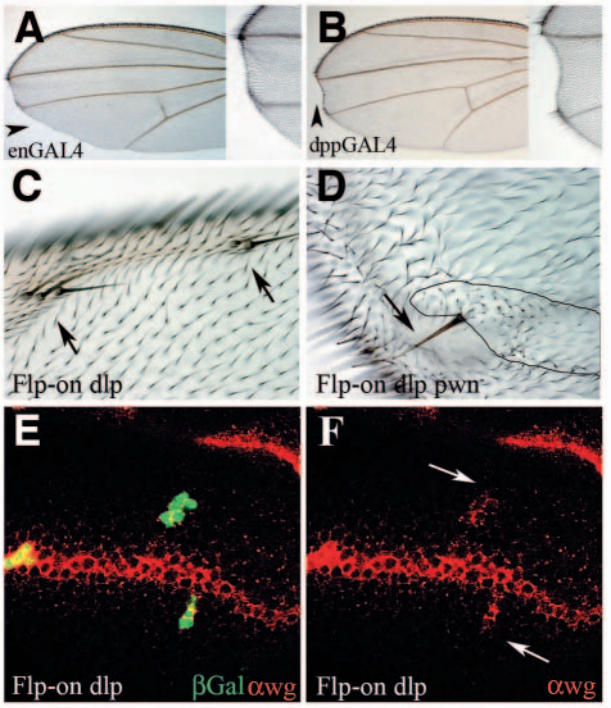

Fig. 5. Cell-autonomous and non-cell-autonomous effects of Dlp overexpression. (A,B) Overexpression of Dlp in broad domains causes the loss of margin tissue. Here, overexpression was activated in the posterior compartment with *engrailed-gal4* (A) or in the central region of the wing with *dpp-gal4* (B). Notching is localised to where the margin overlaps with the region of overexpression (arrowheads). Details of the margin are shown on the right-hand side. (C,D) Overexpression of Dlp in scattered clones (Flp-on Dlp) leads to the formation of ectopic bristles (also to loss of margin tissue, not shown here). (C) Ectopic bristles (arrows) caused by random unmarked clones. (D) Margin area near a clone marked with the *pwn* mutation (outlined). An ectopic bristle (*pwn*$^{+}$, i.e. outside the clone) is seen at the edge of the clone (arrow). (E,F) Overexpression of Dlp causes local accumulation of Wingless at the cell surface. Overexpression was induced in clones marked by β-galactosidase (E). Wingless accumulation is seen in F (visualised by anti-Wingless antibody) (white arrows).

Overexpression of Dlp activates Wingless signalling in a non-cell-autonomous manner

In order to further investigate the specific roles of Dally and Dlp in Wingless activity, we turned to overexpression experiments. When Dally is overexpressed at 25°C with the *dpp-gal4* driver, which is active in a broad group of cells along the anteroposterior boundary in the wing, no apparent effect on wing margin morphology can be seen (see also Strigini and Cohen, 2000). By contrast, overexpressed Dlp induces clear-cut phenotypes. Overexpression of Dlp in broad domains with *engrailed-gal4* or *dpp-gal4* at 25°C mainly causes loss of margin tissue, i.e. a reduction of Wingless signalling there (Fig. 5A,B) (see also Baeg et al., 2001). Likewise, scattered clones of misexpressing cells induced with the 'Flp-on' system (Pignoni and Zipursky, 1997) cause margin loss (not shown). Oddly, however, an opposite phenotype is also seen. As shown in Fig. 5C, the presence of random clones of cells that overexpress Dlp causes the formation of ectopic margin bristles. Under all these experimental conditions, Dlp overexpression induces a strong accumulation of Wingless at the cell surface (only shown here for overexpression in clones, in Fig. 5E,F) (see also Baeg et al., 2001). The induction of opposite phenotypes (loss and gain of Wingless signalling) by overexpression could be explained if Dlp had distinct cell-autonomous and non-cell-autonomous effects. To investigate this further, mitotic clones of Dlp-misexpressing cells were marked with the *pwn* mutation, which affects the morphology of hairs and bristles (Lawrence et al., 2002). As shown in Fig. 5D, ectopic margin bristles are formed by wild-type cells located next to misexpressing cells. This suggests that overexpressed Dlp causes non-cell-autonomous increase in Wingless signalling. At the same time, it appears that Dlp overexpression causes cell-autonomous reduction of Wingless signalling as overexpression in a broad band of cells leads to loss of margin tissue.

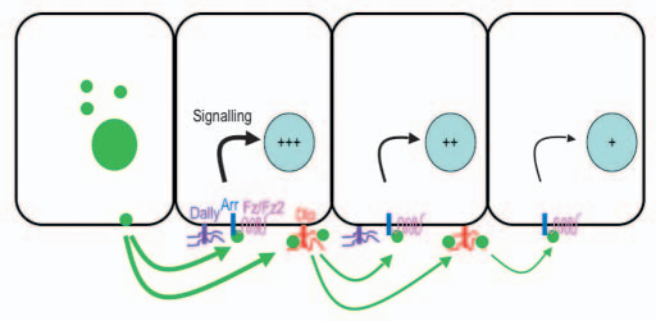

Fig. 6. Model that reconciles the opposite phenotypes seen as a result of *dlp* overexpression. Wingless can bind to several receptors as it reaches a target cell. Binding to Dlp would prevent access to the signalling receptors and favour presentation to a neighbouring cell. By contrast, binding to the signalling receptors would not only lead to signalling but also to trapping and degradation, thus preventing subsequent transport.

Dlp overexpression reduces Wingless signalling near the margin while extending the range of low level signalling

The results described above lead to a model whereby, at the surface of a given cell, Dlp would divert oncoming Wingless away from local signalling while at the same time favouring export or presentation to neighbouring cells (Fig. 6). According to such a model, overexpressed Dlp would be expected to extend the range towards more distant cells while at the same time reducing signalling near the source. This prediction is indeed borne out. Within cells that overexpress Dlp, *senseless*, a gene that responds to high level of Wingless (Nolo et al., 2000), is repressed as expected from the loss of margin tissue. For example, in discs overexpressing Dlp under the control of *engrailed-gal4*, *senseless* expression is abolished in the posterior compartment (right side of the broken line in Fig. 7A). At the same time, the domain of expression of *distal-less*, a low level target (Zecca et al., 1996) is broadened (Fig. 7B), suggesting an extension of the range. A similar result is seen in large overexpression clones (Fig. 7C,D). In this figure, the slight upregulation of *distal-less* in cells flanking the misexpressing clone (arrowhead), consistent with the possibility that

overexpressed Dlp favours presentation of Wingless to neighbouring cells.

Loss of *dlp* activity reduces the range of Wingless

If wild-type Dlp is indeed involved in shunting Wingless to neighbouring cells, one expects that loss of *dlp* activity would reduce the range of Wingless. This is indeed the case. Clones of *dlp* mutant cells contain reduced amount of Wingless in receiving tissue (arrowhead in Fig. 7F) without an apparent reduction at the source. A reduction of Wingless present at the surface of *dlp* mutant cells is also seen (Fig. 7G,H). To assess the functional consequence of reduced Wingless, we looked at the expression of *distal-less* in imaginal discs obtained from homozygous dlp^{MH20} larvae. The domain of *distal-less* expression in *dlp* mutants is significantly narrower than that in wild-type discs, suggesting a reduction of the range (Fig. 7I,J). A concomitant increase in *senseless* expression is seen (Fig. 7K,L) suggesting increased signalling near the source (as expected from ectopic margin bristles, Fig. 4D). Thus, in *dlp* mutants, the balance between short-range and long-range signalling is upset, with short-range signalling being favoured at the expense of long-range signalling.

Both Dally and Dlp bind Wingless in cell culture even though only Dlp overexpression causes Wingless accumulation in imaginal discs

Because *dally* mutants have reduced Wingless signalling, Dally could act as a classical, though non-essential, co-receptor (Ruoslahti and Yamaguchi, 1991), perhaps capturing the ligand and presenting it to the signalling receptors. To determine whether Dally does indeed promote binding of Wingless to the cell surface, we used a tissue culture assay. S2 cells were transfected with a plasmid encoding Dally. After a suitable time to allow expression, cells were transferred to 4°C to prevent endocytosis and treated with conditioned medium containing GFP-Wingless. As shown in Fig. 8A,B, Dally expression causes a significant increase in the accumulation of GFP-Wingless at the surface of S2 cells. Transfection of *dlp*

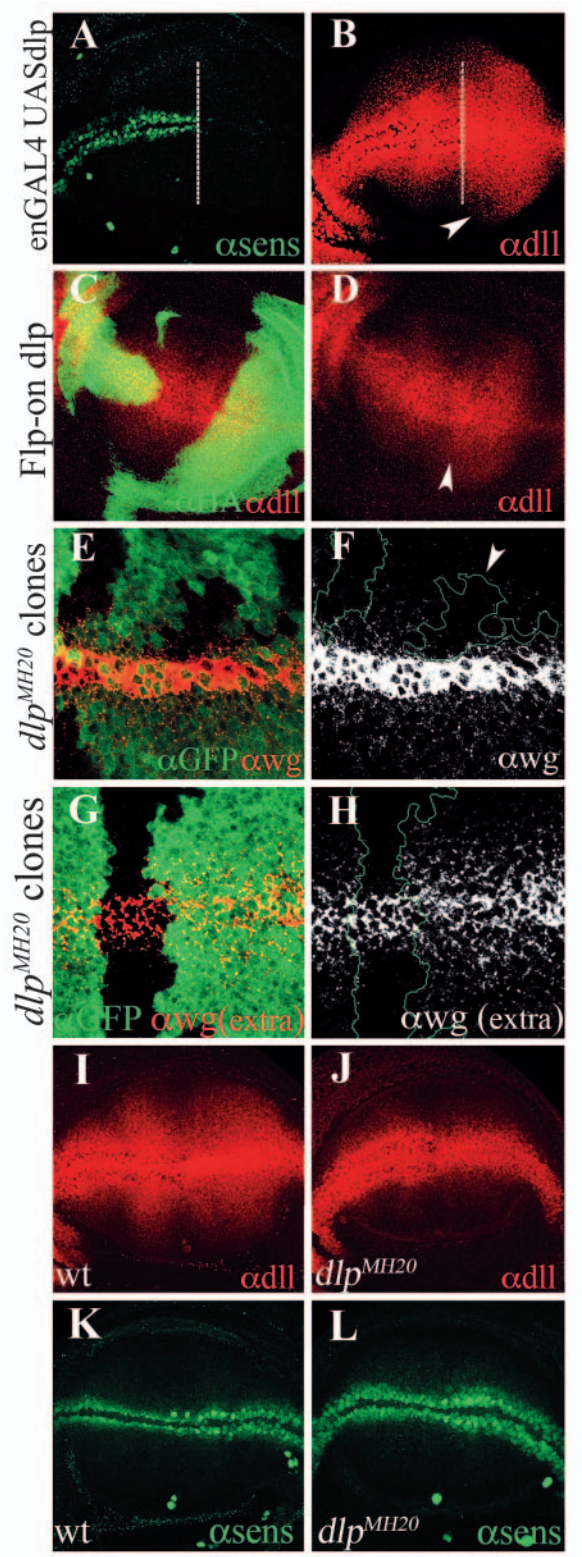

Fig. 7. Dlp overexpression blunts the Wingless gradient while loss of *dlp* sharpens it. (A) Overexpression of Dlp with *engrailed-gal4* eliminates expression of *senseless* (a 'high Wingless target') in the posterior compartment (on the right of the broken line where *engrailed-gal4* is expressed). (B) At the same time, expression of *distal-less*, a 'low Wingless target' is broadened (arrowhead) specifically in the posterior compartment. (C,D) A similar broadening of *distal-less* expression is seen in large Dlp misexpression clones (right-hand clone, marked with GFP in C). There is slight upregulation of *distal-less* at the edge of the clone within its normal domain of expression (arrowhead in D) consistent with increased presentation activity as a result of Dlp overexpression. (E,F) Mutant dlp cells have reduced levels of Wingless protein. Mutant clones are marked by the absence of GFP (green in E). Reduction of Wingless protein (shown in F) is subtle but unambiguous [see, for example, the reduction in the number of vesicles in the mutant area (arrowhead)]. (G,H) Reduction of Wingless protein at the surface of *dlp* mutant cells. Extracellular staining (shown in H) was performed as described previously (Strigini and Cohen, 2000). Again, mutant cells are marked by the absence of GFP (green in G). (I,J) Expression of *distal-less* in wild type (I) and homozygous dlp (J) discs. Both panels are from discs processed and photographed under identical conditions. The domain of *distal-less* expression is clearly narrower in wing discs obtained from *dlp* homozygous larvae than in the wild type. Thus, in *dlp* mutants, a low level target is activated over a reduced range. (K,L) Expression of *senseless* in wild-type (K) and homozygous *dlp* (L) discs. Again, both panels are from discs processed and photographed under identical conditions. The domain of *senseless* expression is slightly wider in the *dlp* mutant, consistent with the formation of ectopic bristles near the adult wing margin.

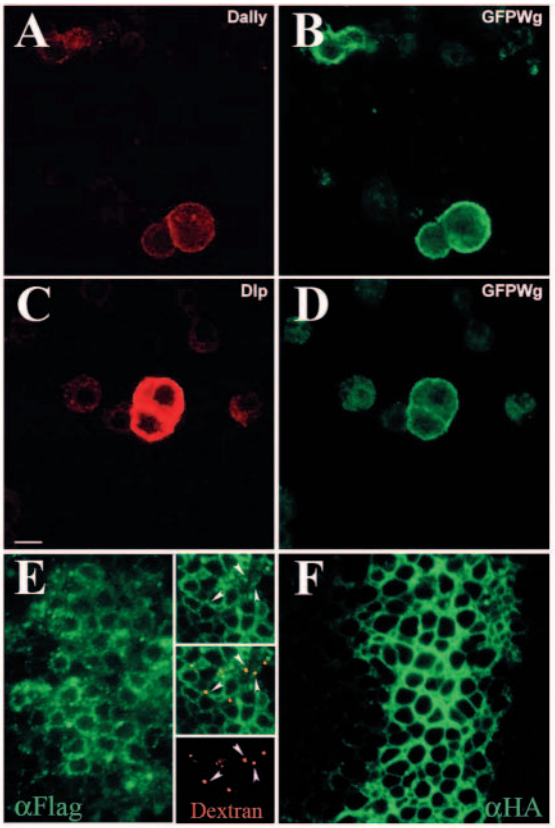

Fig. 8. Binding activity and subcellular localisation of Dally and Dlp. (A-D) Transfection of Dally-HA or Dlp-HA in S2 cells causes accumulation of exogenous GFP-Wingless at the cell surface. Transfected cells are recognised with anti-HA (in A and C). HA immunoreactivity is reproducibly lower for Dally than for Dlp (compare A with C) but we do not know whether this is due to differences in expression levels or epitope accessibility. Nevertheless, Dally transfected cells reproducibly accumulate more GFP-Wingless (compare B with D). (E,F) Subcellular distribution of exogenous Dally and Dlp in wing imaginal discs. FLAG-tagged Dally expressed under the control of *dpp-gal4* is present both at the cell surface and in vesicles (E), while HA-tagged Dlp is almost exclusively seen at the cell surface. Inset in E shows the detail of a disc expressing FLAG-Dally that was briefly stained with Texas Red Dextran to label the endocytic pathway. The disc was immersed live in a solution of Texas Red dextran for 10 minutes. This was followed by a 20 minute chase and subsequent fixation. Partial colocalisation of Dally (green) with dextran (red) shows that some Dally is in endocytic structures.

also causes Wingless accumulation at the cell surface, possibly to a lesser extent (Fig. 8C,D). We suggest that in imaginal discs, after recruitment, Dally could present Wingless to signalling receptors expressed in the same cell. This would be followed by activation of signalling and rapid degradation. In imaginal discs, although exogenous Dlp accumulates almost exclusively at the cell surface, Dally is present both at the cell surface and in vesicles, perhaps because it is continuously endocytosed (insets in Fig. 8E).

Conclusion

The fact that mutations in *dally* and *dlp* cause different phenotypes suggests that, although they both underpin Wingless function, these two glypicans could perform distinct activities. It is likely that both Dally and Dlp are able to capture Wingless at the surface of imaginal disc cells. From the point of view of a given cell in vivo, Wingless captured by Dally would be mostly destined for 'internal consumption', while Dlp-bound Wingless would be for export only. Subsequent long-range transport would occur by hopping from Dlp on one cell to Dlp on the next. Both glypicans would contribute to increasing the concentration of Wingless at the cell surface (Dally in cis and Dlp in trans). We suggest that in the embryo too, Dlp and Dally help in the presentation and reception of Wingless, respectively. However, in this system, little Wingless transport takes place (Pfeiffer et al., 2000), maybe because release of Wingless from Dlp is not allowed. It is interesting that, in embryos, *dlp* is highly expressed in cells that secrete Wingless. Therefore, the role of Dlp would mainly be to ensure that plenty of Wingless is retained at the surface of Wingless-expressing cells thus allowing sustained short-range signalling. In both the embryonic and disc systems, the genetic redundancy between *dally* and *dlp* could be viewed as follows: reduction of capturing activity in *dally* mutants would be compensated by the 'presentation activity' of Dlp and vice versa. Further cell biological work will be needed to fully explore the specific activities of Dally and Dlp and also to discover how Wingless is transferred from one cell to another during transport, perhaps with the help of enzymes such as Notum/Wingful (Gerlitz and Basler, 2002; Giraldez et al., 2002).

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