The glypican Dally-like is required for Hedgehog signalling in the embryonic epidermis of *Drosophila*

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Summary

The *Drosophila* genes *dally* and *dally-like* encode glypicans, which are heparan sulphate proteoglycans anchored to the cell membrane by a glycosylphosphatidylinositol link. Genetic studies have implicated Dally and Dally-like in Wingless signalling in embryos and imaginal discs. Here, we test the signalling properties of these molecules in the embryonic epidermis. We demonstrate that RNA interference silencing of *dally-like*, but not *dally*, gives a segment polarity phenotype identical to that of null mutations in *wingless* or *hedgehog*. Using heterologous expression in embryos, we uncoupled the Hedgehog and Wingless signalling pathways and found that Dally-like and Dally, separately or together, are not necessary for Wingless signalling. Dally-like, however, is strictly necessary for Hedgehog signal transduction. Epistatic experiments show that Dally-like is required for the reception of the Hedgehog signal, upstream or at the level of the Patched receptor.

Key words: Dally, Wingless, heparan sulphate proteoglycan, signal transduction, RNA interference

Introduction

Animal development is under the control of a handful of signalling pathways that are activated by extracellular ligands, the most prominent activating the Hedgehog (Hh), Wnt, FGF, EGF, TGFβ/BMPs or Notch pathways. The secretion, movement and reception of these extracellular signals is tightly regulated, and recent work has implicated the heparan sulphate proteoglycans (HSPGs) in the regulation of ligand activity (Bernfield et al., 1999; Perrimon and Bernfield, 2000; Selleck, 2000; Turnbull et al., 2001). Two types of evidence indicate that HSPGs play a role in signalling: first, extracellular ligands are often found tightly associated with the cell surface, and this association can be inhibited by heparin, a subclass of heparan sulphate. Second, mutations in enzymes involved in the biosynthesis of heparan sulphate chains impair specific signalling events in vertebrate and invertebrate development. In *Drosophila*, the gene *sugarless (sgl)* codes for an UDP glucose dehydrogenase required for the synthesis of heparan sulphate chains, and mutations in *sgl* disrupt both Wingless (Wg, homologue of Wnt-1) and FGF signalling in the embryo (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997; Lin et al., 1999; Toyoda et al., 2000). Mutations in *sulfateless* (*sfl*), which codes for a N-deacetylase/N-sulphotransferase required for the sulphation of heparan sulphate chains, also disrupts Wg and FGF signalling in embryos (Lin et al., 1999; Lin and Perrimon, 1999; Toyoda et al., 2000). *tout-velu* (*ttv*) codes for a co-polymerase required for the elongation of heparan sulphate chains (Bellaiche et al., 1998; The et al., 1999; Toyoda et al., 2000). *ttv* mutations disrupt the movement of Hh in wing discs, and are thought to disrupt Hh signalling but not Wg or FGF signalling in embryos (Bellaiche et al., 1998; The et al., 1999). More recently, mutations in *fringe connection* (*frc*), a gene coding for a UDP sugar transporter, were shown to disrupt Wg or Hh signalling in embryos, as well as FGF signalling (Selva et al., 2001; Goto et al., 2001). Given their pleiotropic effects, however, it is difficult to use these mutants to determine at which step of a given signalling pathway HSPGs intervene.

One avenue of research is to identify the protein cores of HSPGs that are required for each signalling pathway. Two types of proteoglycans bear the majority of heparan sulphate chains at the cell surface: the syndecans and the glypicans (Bernfield et al., 1999). Mutations in the latter produce developmental defects in mice, zebrafish and *Drosophila*, making glypicans good candidates for having a role in signalling (Bernfield et al., 1999; Perrimon and Bernfield, 2000; Selleck, 2000; Song and Filmus, 2002). Glypicans are glycosylphosphatidylinositol (GPI)-anchored proteins, which are thought to be permanently glycanated, and that carry several heparan sulphate chains linked to serine residues adjacent to the plasma membrane. The *Drosophila* genome contains two glypicans: *dally* and *dally-like (dlp).* Dally has been implicated in the regulation of Wg and Dpp signalling, and Dlp in the regulation of Wg signalling (Jackson et al., 1997; Lin and Perrimon, 1999; Tsuda et al., 1999; Baeg et al., 2001).

We have tested the requirement of the two *Drosophila* glypicans for Wg and Hh signalling in the embryonic epidermis. Wg is a secreted glycoprotein that activates the receptors Frizzled and Frizzled2, which then turn on a downstream signalling cascade leading to the activation or repression of target genes (Wodarz and Nusse, 1998). The full-length Hh protein undergoes an autocatalytic processing in the secreting cells and is further modified by addition of two lipids,

a cholesterol and a palmitoyl moiety. Hh binds its receptor Patched (Ptc) on the receiving cells, and this relieves Ptc inhibition on another transmembrane protein, Smoothened (Smo), which in turn transduces the Hh signal (Ingham and McMahon, 2001). Wg and Hh are expressed in stripes in the embryonic segments and their functions in epidermal patterning are now well understood (Martinez Arias, 1993; Hatini and Dinardo, 2001; Sanson, 2001). Early in embryogenesis, Wg is required to maintain the transcription of *engrailed* (*en*) in adjoining cells. Once the expression of *en* becomes independent of Wg, around stage 11, Wg is required for the specification of cells that secrete a smooth or 'naked' cuticle, through the repression of the gene *shavenbaby*. The *en*-expressing cells secrete Hh, which in turn is required to maintain the transcription of *wg*, its main target in the epidermis. Thus *wg*, *en* and *hh* form a regulatory loop in the embryonic epidermis and loss of Wg or Hh signalling leads to the loss of expression of all three genes, generating an identical phenotype at the end of embryogenesis. This characteristic segment polarity phenotype results from the simultaneous loss of polarity within each segment and the loss of naked cuticle (Fig. 1). Importantly, embryos without maternal and zygotic *sgl*, *sfl*, *ttv* or *frc* exhibit this phenotype (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999; The et al., 1999; Selva et al., 2001; Goto et al., 2001). Also, RNA interference (RNAi) against *dlp* or *dally* generates embryos with weak segment polarity phenotypes (Lin and Perrimon, 1999; Tsuda et al., 1999; Baeg et al., 2001). Because these phenotypes can result from either a loss of Hh or Wg, it has been difficult to analyse the requirement of these genes in embryonic patterning. To circumvent this problem, we used heterologous expression to uncouple Wg and Hh signalling in the embryonic epidermis, and tested the requirement of the *Drosophila* glypicans for these two pathways. Since no null mutations are available for *dally* and *dlp*, we took advantage of RNA interference to silence the function of both genes in embryos.

Materials and methods

Drosophila strains

The w^{1118} strain was used as wild type. wg^{CX4}, hh^{AC} and ptc^{IIW} (synonym of ptc^{16}) are null alleles (see FlyBase: http://gin.ebi.ac.uk:7081/).

We used the UAS/Gal4 system to drive ectopic expression (Brand and Perrimon, 1993). All overexpression experiments were carried out at 25°C. The transgenic strains used were: armGal4[11], armGal4<FRT<VP16 and KB19 (Sanson et al., 1996), simGal4 (Golembo et al., 1996), UASwg (Lawrence et al., 1996), UAShh (Fietz et al., 1995), UAShh-N (Porter et al., 1996), UASdlp (Baeg et al., 2001), UASnuclacZ (Mark Muskavitch, Indiana University, USA), ftzlacZ (Jean Paul Vincent, Mill Hill, UK).

Genotypes of embryos are as follow: armGal4; UASwg (Fig. 3B,C), simGal4; simGal4/UASwg (Fig. 3D-F), armGal4; UAShh (Fig. 4B,C), simGal4/UASnuclacZ; simGal4 (Fig. 4D), simGal4; simgal4/UAShh (Fig. 4E,F), armGal4; UAShh-N (Fig. 5A,C), UAShh-N; armGal4; hh^{AC}/hh^{AC}.ftzLacZ (Fig. 5B) UAShh-N; simGal4; simGal4 (Fig. 5D,F), UAShh-N; simGal4; hh^{AC}/hh^{AC}.ftzlacZ (Fig. 5E), armGal4VP16/UASdlp (Fig. 6A), enGal4; UAShh (Fig. 6B), enGal4; UAShh-N (Fig. 6C), ptc^{IIW}/ptc^{IIW} (Fig. 6D,E), ptc^{IIW}/CyOftzlacZ (Fig. 6F).

UAShh, UAShh-N (first and third chromosome insertions) and UASdlp are homozygous stocks. UASwg is a heterozygous stock balanced over TM3hblacZ or TM3actGFP and *lacZ* or *GFP* expression was used to genotype the embryos. ptc^{IIW} homozygous embryos laid by ptc^{IIw}/CyOftzlacZ flies were identified by the absence of *lacZ* expression.

RNA interference

For the synthesis of dsRNA, small regions of the plasmids pBS(KS)-dlp (Baeg et al., 2001) and pBS(KS)-dally (Nakato et al., 1995) were amplified by PCR with primers pairs containing a T7 promoter sequence at the 5′ end (5′TAATACGACTCACTATAGG3′). The PCR products were used as templates for T7 transcription reactions with the Ribomax Large Scale Production kit (Roche). In these reactions, the two strands of RNA self-anneal. The dsRNA was extracted with phenol/choloroform, precipitated with ethanol and resuspended in injecting buffer. The concentration of dsRNA was evaluated on 1% agarose gel before and after extraction, and the volume of injection buffer adjusted to have a final concentration of about 5 μg/μl. Two dsRNA were prepared for silencing *dlp* (CG32146, NCBI accession noAE003554): 326 nucleotides (nt) from position 169 (primers 5′ACCATGTTGCACTTCAA3′ and 5′CTGCAATGCAGATGTTGT3′), 364nt from position 37772 (primers 5′AAGAATCCGCTCATCCACAC3′ and 5′AATTTTGGACTCGCATTTCG3′). Three dsRNA were prepared for silencing *dally* (CG4974, NCBI accession noAE003533): 333nt from position 372 (primers 5′GCTCTCTCTTCGACCACCAC3′ and 5′CAGACACAGTGGATGATGGG3′), 394nt from position 59157 (primers 5′TGACTTGCACGAGGACTACG3′ and 5′ATGGGTGGTGACCAGATTGT3′), and 303nt from position 61246 (primers 5′TAGCCAGCGATATAATCCCG3′ and 5′GACTCCACTTCGTTGGTGGT3′). All sequences were submitted to BLAST and chosen to minimise the homology with other sequences. Injections were initially performed with each sequence to ensure that the results obtained were reproducible (see Table 1). The 3′ dsRNA sequences for both *dlp* and *dally* were then used for subsequent RNAi experiments. For injection of both dsRNA (Fig. 3F), 5 μg/μl *dally* 3′ dsRNA was mixed in a 1:1 ratio with 5 μg/μl *dlp* 3′ dsRNA.

For injections, 2- to 4-day old synchronised flies were left to lay for 30 minutes at 25°C. Embryos were dechorionated in 50% commercial bleach and aligned on coverslips onto a strip of heptane glue. Embryos were then desiccated, covered with Voltalef oil and injected at the posterior end. All these steps were done at 19°C and did not last more than 30 minutes to ensure that embryos were injected at early blastoderm, to minimise injection defects. After injection, embryos were examined under the dissecting microscope, and cellularised, unfertilised or damaged embryos were eliminated. w^{1118}-injected embryos were left to develop at 18°C, whereas embryos carrying UAS/Gal4 transgenes were left to develop at 25°C. Cuticle preparations were standard (see below) but fixation of injected embryos for immunochemistry or in situ hybridisation was done as follow (Vincent and O'Farrell, 1992): Under the dissecting microscope, the Vortalef oil was removed as much as possible around the embryos using a razor blade. Embryos were then detached from the coverslip using heptane, transferred to a fixative solution of 10% formaldehyde in PBS, and left to fix for 35 minutes. The fixation was longer than standard protocols because of the presence of oil around the embryos. Fixed embryos were transferred to a Petri dish and devitelinised by hand using a sharp needle. The embryos were then dehydrated and stored in 100% methanol at –20°C.

RT-PCR

The RNAi efficiency was estimated by measuring endogenous mRNA levels using semiquantitative RT-PCR. Total RNA was isolated from batches of 60 injected embryos, after washing them with heptane to remove the Voltalef oil. The large number of embryos used in an extract was to ensure that the whole range of phenotypes observed with RNAi would be represented. Embryos were homogenised in 300 μl Trizol + 0.2 μg Linear Polyacrylamide as a carrier (Sigma),

followed by chloroform extraction and isopropanol precipitation. Extracted RNA was resuspended in 30 µl water, and 10 µl were used for reverse trancription, priming with oligo(dT). Reverse transcription reactions (20 µl) contained 10 µl RNA extract, 1 µl oligo(dT) 10 µM, 4 µl Superscript buffer, 2 µl DTT 0.1 M, 2 µl dNTPs 10 mM, 0.5 µl RNasout and 0.5 µl Superscript Reverse Transcriptase (all reagents are from Invitrogen, unless specified). After incubation for 1 hour at 42°C, the reactions were stopped by heating for 10 minutes at 65°C. PCR reactions (50 µl) used 1 µl of the reverse transcription reaction (which corresponds to 1 embryo equivalent of cDNA) and contained 5 µl 10× PCR buffer, 1 µl dNTPs 20 mM, TaqPlus polymerase 0.5 µl (Stratagene) and 2.5 µl of each primer (10 µM). Samples were denaturated for 2 minutes at 94°C before cycling 30 times 1 minute at 55°C, 1 minute 30 seconds at 72°C and 30 seconds at 94°C. Primers used were: 5′AGCAAAACAATCGCGACG3′ and 5′GCCATTTGAGCTGTTTGC3′ for *dlp* (301nt product from positions 49658 to 59016). and 5′ATTTGCGGCGGAAACTG3′ and 5′TGGCCATTGCTGTTCGTA3′ for *dally* (305nt product from positions 37011 to 37354). 10 µl of each PCR reaction were resolved on 1% agarose gel stained with ethidium bromide, and quantification was done using the AlphaImager imaging system. Reactions with increasing numbers of cycles were initially run to determine the geometric phase: we could detect a geometric increase of PCR products between 25 and 35 cycles for both primer pairs, thus we used 30 cycles for subsequent RT-PCR reactions.

Embryo preparations

For in situ hybridisation, embryos were fixed and hybridised with digoxigenin or fluoresceine-labelled single stranded RNA probes as described by Jowett (Jowett, 1997), except that no proteinase K treatment was performed. *wg*, *en* and *rho* cDNAs were a gift from J. P. Vincent, and *lacZ* a gift from V. Morel.

Immunochemistry was done according to standard protocols. Primary antibodies used were mouse anti-En (1:50) (Hybridoma Bank) and rabbit anti-Dlp (1:50) (Baeg et al., 2001).

For cuticle preparations, embryos were mounted in Hoyer's/lactic acid (1:1) and visualised with dark-field microscopy.

Results

RNAi silencing of *dally-like* but not *dally* generates a full segment polarity phenotype in the embryonic epidermis

To block *dally* or *dally-like* (*dlp*) function, we injected syncytial embryos with dsRNA fragments about 300 nucleotides long, which corresponded either to the 5′ or the 3′ end of the *dlp* mRNA or to the 5′, middle or 3′ of the *dally* mRNA (Fig. 1D). We allowed injected embryos to develop until the end of embryogenesis and examined their cuticle patterns (Fig. 1). A fraction (14%, *n*=99) of control embryos that were injected with buffer alone exhibited weak segmentation defects (Table 1 and not shown). These defects consist of fusions or deletions of denticle belts, which are distinct from transformation of naked cuticle to denticles seen in segment polarity mutants. They indicate a loss of tissue, and are presumably a consequence of the injection process. Injection of 3′, middle or 5′ *dally* dsRNA at a concentration of 5 µg/µl, generates weak segmentation phenotypes identical to those of buffer-injected embryos, with the same frequency (15%, *n*=226) (Fig. 1F and Table 1). By contrast, injection of either the 3′ or 5′ *dlp* dsRNA, produces segmentation defects in almost all embryos, and about two-thirds of these exhibit either a strong or a full segment polarity phenotype. We scored as having a full segment polarity phenotype those embryos

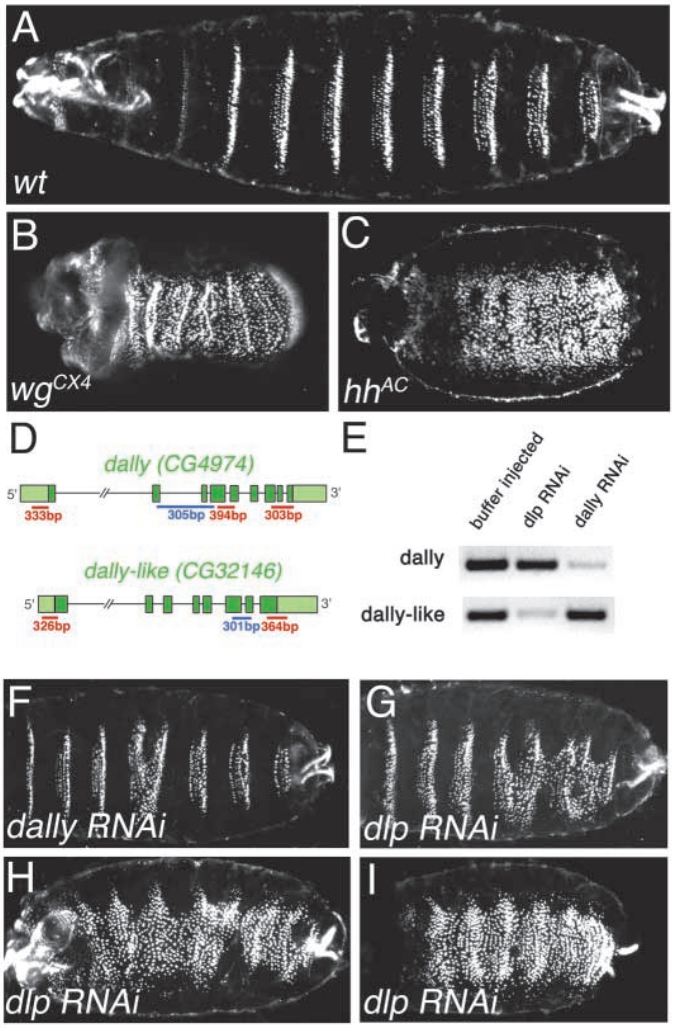

Fig. 1. RNAi silencing of *dally-like* but not *dally* generates a segment polarity phenotype in *Drosophila* embryos. (A) Ventral view of a wild-type larval cuticle. Unless specified, the head of embryos is to the left. Eight belts of denticles are visible in the abdomen. (B and C) Segment polarity phenotype of null mutations in *wingless* (wg^{CX4}) and *hedgehog* (hh^{AC}). The areas of naked cuticle are replaced by denticles, generating a lawn of denticles without clear polarity. (D) Position of the dsRNA sequences (in red) and the RT-PCR sequences (in blue) used in *dally* and *dally-like* RNAi experiments. (E) Agarose gel electrophoresis of semiquantitative RT-PCR reactions performed on extracts of embryos injected by either buffer, *dlp* 3′dsRNA or *dally* 3′dsRNA. There is at least a fourfold decrease in *dlp* mRNA following *dlp* RNAi, and at least a fivefold decrease in *dally* mRNA following *dally* RNAi. By contrast mRNA levels of *dally* and *dlp* following *dlp* and *dally* RNAi, respectively, are identical to the levels after injection of buffer. (F) Weak segmentation defects exhibited by *dally* dsRNA-injected embryos. The same type of defects are found in buffer-injected embryos (not shown), and thus are a consequence of the injection process. There is no clear transformation of naked cuticle into denticles in these embryos, but rather a loss of tissue that leads to the fusion of denticle belts. (G-I) Segment polarity defects found in *dlp* dsRNA injected embryos. (G) The weak segment polarity phenotypes are distinct from the phenotypes observed in buffer and *dally*-injected embryos: the naked cuticle is transformed into denticles without much tissue loss. (H) Strong segment polarity phenotype and (I) lawn of denticles identical to the phenotype of *wingless* or *hedgehog* null mutants. (See also Table 1.)

Table 1. Distribution of the segmentation defects in embryos injected with *dally* or *dally-like* dsRNA

Treatment	*n*	Segmentation defects (total)	Weak segmentation defects	Strong segmentation defects	Lawn of denticles
Buffer	99	14 (14%)	14 (14%)	0	0
dally RNAi	226	35 (15%)	35 (15%)	0	0
dally-like RNAi	211	193 (91%)	60 (28%)	46 (22%)	87 (41%)

Embryos were injected with either buffer, *dally* or *dally-like* dsRNA, and the cuticle of the ventral abdomen scored for segmentation defects at the end of embryogenesis. Only embryos with an intact cuticle in the trunk were analysed (*n*=total number of analysed embryos). Segmentation defects were classified into three categories. In 'lawn of denticles' and 'strong segmentation defects', every segment had transformations of naked cuticle into denticles, with some naked cuticle remaining in embryos of the 'strong segmentation defects' category. The other embryos with segmentation defects were categorised as weak. Only weak segmentation defects were found after injection of *dally* dsRNA or buffer. *dally* RNAi was performed using the 5′, middle or 3′ dsRNA sequences. The same proportion of weak segmentation defects were observed in each case and the results have been added together for this table. For *dally-like*, injections were performed with a 5′ dsRNA or a 3′dsRNA. The same distribution of weak, strong and lawn phenotypes were observed (not shown). The table shows the result of a typical experiment using the 3′ dsRNA sequence, which is the dsRNA used for the RNAi experiments presented in Figs 2-6.

showing a lawn of denticles ventrally, with no naked cuticle left (Fig. 1I). Embryos with all segments affected, but with naked cuticle patches remaining were scored as strong segment polarity phenotypes (Fig. 1H). The rest of the embryos were classified as having weak segmentation defects (Fig. 1G), although most of these embryos showed stronger defects than in *dally* dsRNA injection or buffer injection (Fig. 1F and not shown). Table 1 shows the distribution of phenotypes for a typical experiment using the *dlp* 3′ dsRNA sequence: 91% of injected embryos showed segmentation defects, and 63% exhibited a strong or full segment polarity phenotype (*n*=211). Both 3′ and 5′ sequences gave the same distribution of phenotypes (data not shown).

To check the efficiency of the RNAi, we used semiquantitative RT-PCR to monitor the levels of *dally* or *dlp* mRNAs in injected embryos. Total RNA was extracted from batches of 60 injected embryos after 7 hours of development (stage 11/12), and the derived cDNAs were amplified by PCR using oligonucleotide pairs targeting a 300 nt sequence just upstream of the 3′ dsRNA sequences (Fig. 1D). RT-PCR on extracts from buffer-injected embryos detected similar mRNA levels for *dally* and *dlp* (Fig. 1E). RT-PCR on extracts from embryos injected with the 3′ dsRNA sequence of *dally* showed a strong reduction of *dally* mRNA as expected, while *dlp* mRNA levels were unchanged (Fig. 1E). The converse is true for RT-PCR on extracts of embryos injected with the 3′ dsRNA sequence of *dlp*: *dlp*, but not *dally*, mRNA levels were strongly reduced (Fig. 1E). This demonstrates that RNAi efficiently silences both *dally* and *dlp*, and also that RNAi directed at one gene does not affect the other.

These results show that RNAi silencing of *dlp* produces a severe and penetrant segment polarity phenotype. Since *dlp* mRNA is produced maternally as well as zygotically (Khare and Baumgartner, 2000), the strength of the phenotype suggests that injection of dsRNA inactivates both pools of mRNA. The full phenotype is identical to *wg* or *hh* loss-of-function phenotypes in embryos (compare Fig. 1I with B,C). Previous work reported weaker segment polarity phenotypes in the cuticle following *dlp* RNAi (Baeg et al., 2001). A possibility is that the use of small dsRNA sequences (300-400nt) in our study, rather than dsRNA corresponding to larger parts of the gene, allowed us to inject higher concentrations of dsRNA, and as a consequence to obtain stronger phenotypes.

In contrast to previous reports (Lin and Perrimon, 1999; Tsuda et al., 1999; Baeg et al., 2001), we did not obtain any segment polarity phenotypes with *dally* RNAi, despite testing three different dsRNA sequences and using a high concentration of dsRNA. Furthermore, the RT-PCR controls show that *dally* had been efficiently silenced in our experiments. A possible explanation for this discrepancy is that, in previous studies, the use of a dsRNA corresponding to large sequences of *dally* had resulted in partial silencing of *dlp*, because of small regions of sequence homology.

dally-like RNAi mimics *hedgehog* loss of function in the embryonic epidermis

All mutations that give strong segment polarity phenotypes disrupt either Wg or Hh signalling, leading to a loss of *en* expression at mid-embryogenesis. Wg is required to maintain the expression of *en* across the parasegmental boundary, whereas Hh is secreted by the *en* cells, and in turn maintains *wg* expression (Fig. 2E) (Martinez Arias, 1993). In *wg* null mutant embryos, En protein disappears completely from the ectoderm of the trunk by stage 11 (Fig. 2B). In a *hh* null mutant, En starts disappearing at stage 11 and is mostly gone at stage 12 (Fig. 2C,H). In *dlp* RNAi embryos, En starts to be lost at stage 11, and by late stage 12 only patches of En remain in the ectoderm (Fig. 2D,I). The timing of the loss of En resembles that of a *hh* null mutant rather than a *wg* null mutant. In contrast, *dally* RNAi embryos did not show any defects in the pattern of En (data not shown).

We also looked at the pattern of *rhomboid* (*rho*) transcription in RNAi *dlp* embryos. In wild-type embryos, *rho* is expressed in a single stripe posterior to the *en* domain (Fig. 2K), because it is activated by Hh and Serrate (Ser) signalling and repressed by Wg signalling (Alexandre et al., 1999) (Fig. 2O). In *wg* mutants, two stripes of *rho* expression were found on both sides of the cells that were expressing *en* (Fig. 2L). In contrast, only one stripe of *rho* expression was found in *hh* mutants as in wild type, presumably because there is enough Wg activity left to repress the anterior stripe, while part of the posterior stripe was maintained by Ser signalling (Fig. 2M). In embryos injected for *dlp* dsRNA, we found only one stripe of *rho* expression, as in *hh* mutants (Fig. 2N). Taken together, these results suggest that loss of *dlp* mimics *hh* loss of function rather that *wg* loss of function.

RNAi silencing of *dlp* and *dally* do not inhibit Wg signalling

To uncouple the regulatory loop between Wg and Hh signalling in embryos, we expressed UASwg under the control of two Gal4 drivers, armadillo-Gal4 (armGal4) and singleminded-Gal4 (simGal4), and looked at two different targets of Wg signalling: the transcription of *en* and the specification of naked cuticle. ArmGal4 drives expression ubiquitously (Sanson et al., 1996), and simGal4 drives expression in the ventral midline (Golembo et al., 1996). As Wg specifies naked cuticle in most

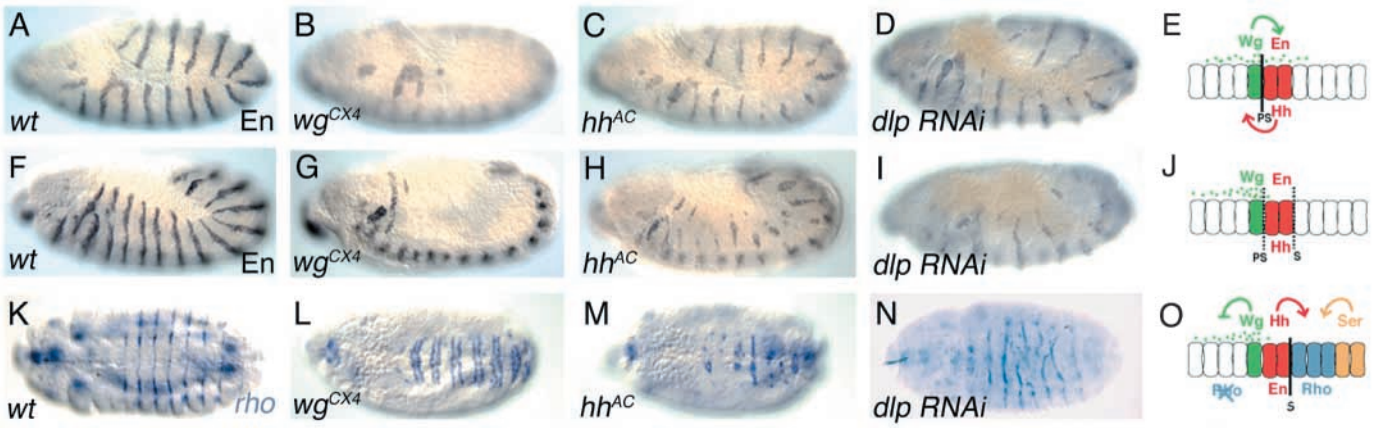

Fig. 2. *Engrailed* and *rhomboid* expression in *dally-like* RNAi embryos. (A-D) Antibody staining against En in stage 11 embryos. Wild-type En expression (A). En expression in the ectoderm is completely gone in a *wg* null mutant (B), and has just started to fade in a *hh* null mutant (C) and *dlp* RNAi embryos (D). (E) Schema representing the autoregulatory loop between *wg*, *en* and *hh* at stage 9-10 in the ectoderm. As a consequence of this loop, En is lost earlier in *wg* mutants than in *hh* mutants. Note that at this stage, the gradient of Wg protein is symmetrical. PS: parasegment boundary. (F-I) Antibody staining against En in stage 12 embryos. En expression is mostly gone in a *hh* null mutant (H) and *dlp* RNAi embryos (I). Mutant phenotypes were found in *dlp* RNAi embryos in 87% of cases (n=140). (J) Schema showing the segmental gene regulation at stage 11-12. At stage 11, En expression becomes independent of Wg, and the Wg gradient becomes asymmetrical. The parasegment groove (PS) disappears around stage 12 when the segment boundary (S) groove starts to be visible. (K-N) In situ hybridisation against *rhomboid* (*rho*) in stage 14 embryos. In wild type, *rho* is expressed in one stripe per segment (K), whereas two stripes per segment are found in the *wg* null mutant (L). In *dlp* RNAi embryos (75%, n=24) (N), as in a *hh* null mutant (M), one irregular stripe is found per segment as in wild type, suggesting that loss of *dlp* mimics the loss of *hh*. (O) Schema depicting the intrasegmental patterning occurring in stage 12-14 embryos. *Wg* represses *rho* expression on the anterior side of the Engrailed domain, whereas *hh* and *serrate* (*ser*) maintain *rho* expression on the posterior side. S, segment boundary.

segments through the repression of the zinc-finger transcription factor *shavenbaby* (Payre et al., 1999), the secretion of naked cuticle gives a convenient read-out of Wg signalling activity (Lawrence et al., 1996; Sanson et al., 1999).

In armGal4/UASwg embryos, *en* transcription was enlarged from a stripe about two cells wide to a stripe spanning about half a segment, which coincided with the domain of competence for *en* transcription (Fig. 3A,B). In armGal4/UASwg [dlp RNAi] embryos, *en* ectopic expression is unaffected (Fig. 3C). Expression using simGal4 allows the examination of non cell-autonomous signalling, because Wg is secreted by the midline cells and acts on the adjoining epidermal cells. In simGal4/UASwg embryos, cells specified naked cuticle in the ectoderm a few cell diameters away on either sides of the midline (Fig. 3D). We could not look at *en* transcription in this experiment because simGal4 expression starts at stage 11, when *en* expression has become independent of Wg signalling. In simGal4/UASwg [dlp RNAi] embryos, naked cuticle specification was unaffected around the midline (Fig. 3E). The epidermis also exhibited a strong segment polarity phenotype lateral to the stripe of naked cuticle, demonstrating that *dlp* has been silenced efficiently and thus providing an internal control for the experiment. Together, the simGal4 and armGal4 experiments suggest that Dlp is not required for Wg signalling in the embryo.

It is possible that Dally and Dlp function redundantly in Wg signalling in embryos. To test this, we injected *dally* and *dlp* 3′ dsRNAs together into simGal4/UASwg embryos. As for the injection of *dlp* dsRNA alone, a segment polarity phenotype was produced in the lateral domain (Fig. 3F). However, the stripe of naked cuticle at the ventral midline was unaffected, showing that silencing both *dally* and *dlp* is not sufficient to inhibit Wg signalling in embryos. As a control, we also injected *dally* 3′ dsRNA into simGal4/UASwg embryos, and found that no segment polarity phenotype could be detected in the lateral domain, as expected, and that the formation of naked cuticle was unaffected at the midline (not shown). These experiments suggest that *dally* and *dlp* are dispensable for Wg signalling in embryos, and that they do not function redundantly in this pathway.

Dally-like is required for Hedgehog signalling

Since *dlp* RNAi gives a strong segment polarity phenotype but does not affect Wg signalling, it is probable that Dlp is required for either Hh transcription or Hh signalling. To test this, we expressed Hh ubiquitously with armGal4, and used the activation of *wg* transcription as a read-out of Hh signalling. In armGal4/UAShh embryos, *wg* expression was enlarged from a stripe one cell wide to a stripe covering about half a segment, which corresponded to the domain of competence for *wg* transcription (Fig. 4A,B). In armGal4/UAShh embryos injected with *dlp* dsRNA, both ectopic and endogenous *wg* expression were lost at stage 11 (Fig. 4C). We also looked at the non cell-autonomous effect of Hh, expressing UAShh under the control of simGal4 (Fig. 4D). In simGal4/UAShh embryos, Hh activated the transcription of *wg* in the ectoderm, a few cell diameters on either side of the midline, within the *wg* competence domain of each segment (Fig. 4E). In simGal4/UAShh [dlp RNAi] embryos, both endogenous and ectopic *wg* expression were lost at stage 11 (Fig. 4F). Since *dlp* RNAi does not affect the activity of the armGal4 and simGal4 drivers (see previous section), these experiments demonstrate that Dlp is required for Hh signalling.

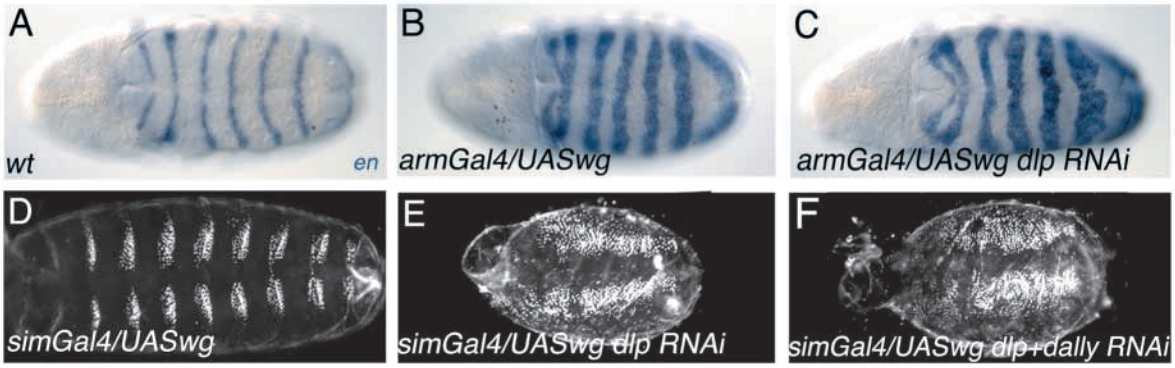

Fig. 3. RNAi of *dally-like* and *dally* do not inhibit Wingless signalling. (A-C) Late stage 11 embryos. *en* transcription revealed by in situ hybridisation in wild type (A) and in embryos ectopically expressing *wg* (B,C). In armGal4/UASwg embryos, the ubiquitous activation of *wg* signalling stimulates *en* transcription in a competence domain spanning half a segment (B). The ectopic transcription of *en* is unaffected in embryos of the same genotype injected with *dlp* dsRNA (*n*=83), showing that *dlp* is not required for Wg signalling (C). (D-F) Cuticle preparations of simGal4/UASwg embryos, where the cells of the ventral midline express *wg*. (D) In response to Wg signalling, the cells in the ventral-most portion of the denticle belts secrete a naked cuticle. (E) In embryos of the same genotype injected with *dlp* dsRNA, a segment polarity phenotype appears in the ventral-lateral portions of the abdomen (87%, *n*=119), showing that *dlp* has been silenced efficiently. However, naked cuticle is still produced at the midline, indicating that ectopic Wg signalling is unaffected by the loss of *dlp*. (F) The same proportion of segment polarity phenotypes is found when *dlp* and *dally* dsRNA are injected together in simGal4/UASwg embryos (81%, *n*=67). As occurs with *dlp* single injection, naked cuticle secretion is unaffected at the midline, showing that these genes are not required in a redundant manner for Wg signalling.

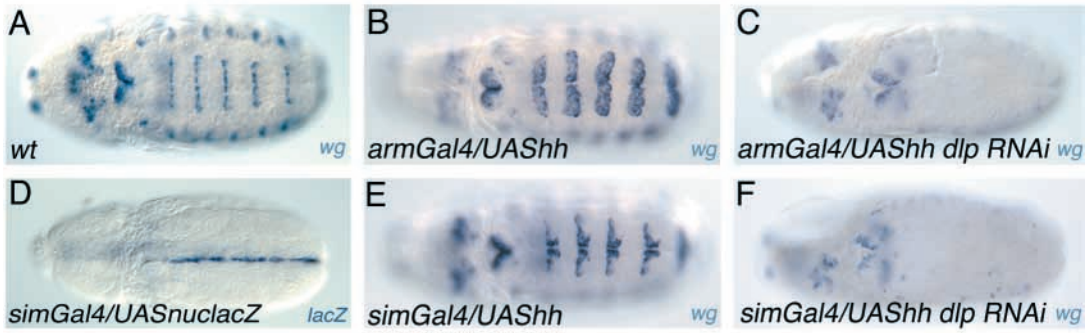

Fig. 4. *Dally-like* is required for Hedgehog signalling. (A-C,E,F) *wg* transcription revealed by in situ hybridisation in wild type (A) and in embryos ectopically expressing *hh* (B,C,E,F). (B) In armGal4/UAShh embryos, *hh* is expressed in every epidermal cells. In response to Hh signalling, *wg* transcription is activated in half a segment, which corresponds to its competence domain. (C) In embryos of the same genotype injected with *dlp* dsRNA, ectopic and endogenous expression of *wg* disappears completely (72%, *n*=47), showing that *dlp* is required for Hh signalling. (D) In situ hybridisation against *lacZ* in simGal4/UASnuclacZ embryos showing that simGal4 drives expression in the ventral midline. (E) In simGal4/UAShh embryos, *hh* is expressed in the ventral midline. In response, *wg* transcription is activated over a few cell diameters on either side of the midline, in each segmental domain of competence. (F) In simGal4/UAShh embryos injected with *dlp* dsRNA, both ectopic and endogenous *wg* expression are gone (97%, *n*=29), confirming the requirement of *dlp* for Hh signalling (F). All embryos shown are late stage 11.

Dally-like is required downstream of Hedgehog processing

Dlp could be required either for Hh secretion from the signalling cells, for Hh movement, or for Hh signal transduction. In the secreting cells, Hh undergoes an intramolecular cleavage which is catalysed by the C terminus of the protein. The liberated N-terminal fragment (the active ligand) is coupled to a cholesterol moiety (Ingham and McMahon, 2001). To determine whether *dlp* is required in the signalling cells, we expressed an engineered form of Hh, Hh-N, which is pre-cleaved and not modified by cholesterol (Porter et al., 1995) and tested whether Hh-N could bypass the requirement for *dlp*. ArmGal4/UAShh-N embryos ectopically expressed *wg* in the same pattern as armGal4/UAShh embryos (compare Fig. 4B and Fig. 5A). SimGal4/UAShh-N embryos ectopically expressed *wg* on both sides of the midline, as in simGal4/UAShh embryos, but over a longer distance from the midline (compare Fig. 4E and Fig. 5D). This is consistent with previous findings that Hh-N moves further in a field of cells than Hh (Burke et al., 1999; Porter et al., 1996). In both armGal4/UAShh-N and simGal4/UAShh-N embryos injected with *dlp* dsRNA, *wg* ectopic expression is completely lost along with *wg* endogenous expression (Fig. 5C,F).

The activity of Hh-N is partially dependent upon the activity of wild-type Hh in embryos (Gallet et al., 2003). To control for this, we overexpressed UASHh-N in a *hh* null mutant background. Most of *wg* ectopic expression remained in armGal4/UAShh-N[hh^{-}] embryos (Fig. 5B), whereas small

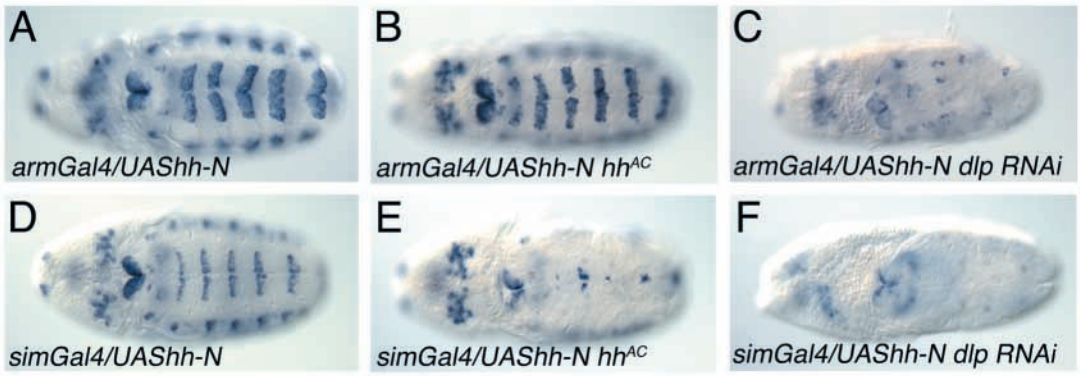

Fig. 5. A pre-processed form of Hedgehog, Hh-N, requires Dally-like for its activity. All panels show *wg* in situ hybridisation in late stage 11 embryos. (A-F) Ectopic expression of a pre-processed, cholesterol unmodified form of Hh, Hh-N. (A) In armGal4/UAShh-N embryos, ubiquitous Hh signalling activates *wg* transcription in the whole competence domain. (B) When the same experiment is repeated in a *hh* null background, ectopic *wg* expression is mostly unaffected. Endogenous *wg* expression is expected to disappear in absence of *hh*, which explains the slightly irregular pattern. (C) In armGal4/UAShh-N injected with *dlp* dsRNA, both ectopic and endogenous *wg* expression disappear (82%, *n*=39), showing that Hh-N requires Dlp for its activity. (D) In simGal4/UAShh-N embryos, Hh-N secretion from the midline activates *wg* transcription on both sides of the midline within each competence domain. (E) In simGal4/UAShh-N [hh$^-$] embryos, Hh-N activates *wg* transcription less efficiently and at short distance from the source, suggesting that Hh-N is partially dependent on endogenous Hh for its non-autonomous activity. However, in simGal4/UAShh-N embryos injected with *dlp* dsRNA (F), all ectopic *wg* transcription is wiped out (93%, *n*=54), showing that Hh-N requires Dlp activity for both its autonomous and non-autonomous effects. Both sets of experiments indicate that Dlp acts downstream of Hh processing.

patches of *wg* ectopic expression remained in simGal4/UAShh-N[hh$^-$] embryos (Fig. 5E). This indicates that Hh-N can still signal in the absence of endogenous Hh, but that Hh-N might be dependent upon Hh for its movement from cell to cell. In contrast, all *wg* expression was lost in armGal4 or simGal4/UAShh-N[dlp RNAi] embryos (Fig. 5C,F), showing that the activity of Hh-N is absolutely dependent on Dlp. We conclude that Dlp is required downstream of Hh processing and cholesterol modification.

Increasing the concentration of Dally-like does not increase the range of Hedgehog signalling

To test if Dlp facilitates the movement of Hh in the ectoderm, we overexpressed Dlp in embryos, and assessed the range of Hh signalling by looking at the width of the *wg* stripe (Fig. 6A). Overexpressing UASdlp with either enGal4 or ptcGal4 did not have any effect on *wg* expression and the embryos were viable (data not shown). As a control, we immunostained for the Dlp protein in enGal4/UASdlp embryos, and found Dlp expressed in stripes as expected (not shown). Also, enGal4/UASdlp adult flies exhibited notched wings as previously observed (Baeg et al., 2001) and ptcGal4/UASdlp flies died at the pharate stage (data not shown), thus indicating that UASdlp expressed an active protein. To increase the quantity of Dlp protein made, we used the armGal4VP16 driver, which is the strongest driver available in embryos (Sanson et al., 1996). We could not detect any enlargement of the *wg* stripe in armGal4VP16/UASdlp embryos (Fig. 6A). As a comparison, expressing UAShh in *en* cells did lead to a moderate but detectable enlargement of the *wg* stripe at stage 11 (Porter et al., 1996) (Fig. 6B). Furthermore, overexpression of UAShh-N in the same cells lead to a larger stripe of *wg* expression, consistent with the idea that Hh-N can travel a longer distance than Hh (Porter et al., 1996) (Fig. 6C). We conclude that increasing the concentration of Dlp in embryos does not increase the range of Hh signalling.

Dally-like is required upstream or at the level of the Patched receptor

Since Dlp is not required for Hh processing and cholesterol modification, and does not stimulate Hh movement when overexpressed, it is probable that Dlp is required for the transduction of the signal. In absence of the Patched (Ptc) receptor, Smoothened constitutively activates the Hh intracellular pathway (Ingham and McMahon, 2001). We looked at this constitutive signalling in *ptc* mutants, in the presence or absence of *dlp*, using *wg* transcription as a read-out. In embryos homozygous for the null mutation *ptc^{IIW}*, *wg* transcription was enlarged in a pattern similar to that in armGal4/UAShh embryos (Fig. 6D). When we injected *dlp* dsRNA into *ptc^{IIW}* embryos, most of the homozygous embryos maintained *wg* ectopic expression (Fig. 6E). Three homozygous embryos out of thirty (10%) had partially lost *wg* expression, but close examination showed that loss of expression was always associated with a disrupted epithelium. Thus, in *ptc*- embryos, injection of *dlp* dsRNA does not abolish Hh constitutive signalling. In contrast, *wg* endogenous expression disappeared in 78% of the heterozygous embryos (*n*=58), showing that *dlp* has been efficiently silenced in this experiment (Fig. 6F). We conclude that *ptc* is epistatic to *dlp*. This indicates that Dlp acts either upstream or at the level of the Ptc receptor.

Discussion

Heparan sulphate modifications have been implicated in several signalling pathways during animal development, including the Hh and Wg signalling pathways, based on the phenotype of mutants in enzymes required for heparan sulphate biosynthesis. However, it remains unclear which proteins are modified by these enzymes, and how the modifications affect a given signalling event. Since most heparan sulphate chains at the cell surface are thought to be carried by proteoglycans

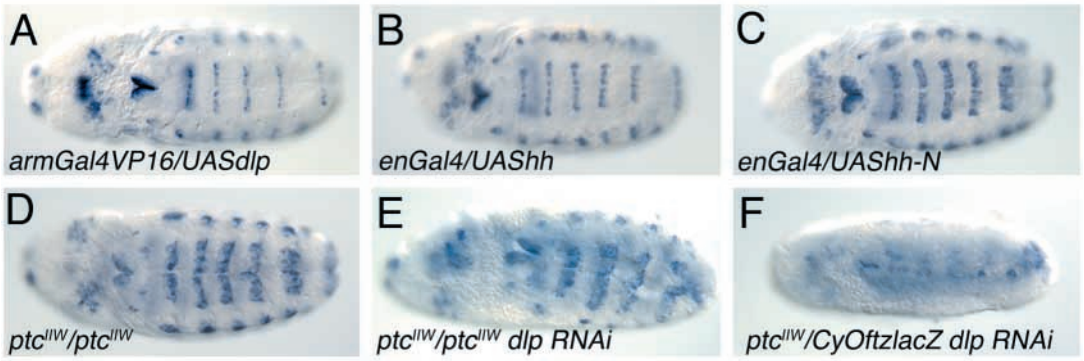

Fig. 6. Dally-like is required upstream or at the level of Patched in the Hedgehog pathway. (A-C) Assays for the range of Hh signalling. (A) ArmGal4VP16/UASdlp embryos ubiquitously express Dlp at high levels, but *wg* transcription is activated in a single row of cells, as in wild type, showing that Dlp does not stimulate the range of Hh signalling in embryos. (B) In contrast, increasing the concentration of wild-type Hh in the cells that normally secrete it (the En cells) stimulates *wg* transcription over two to three cell diameters. (C) Repeating the same experiment with Hh-N leads to the stimulation of *wg* transcription over three to five cell diameters. (D-F) Epistatic relationship between *patched (ptc*) and *dlp*. (D) In *ptc* homozygous null mutant embryos, Hh signalling is constitutively activated and as a result *wg* transcription is stimulated in the whole competence domain. (E) In ptc^{-} homozygous embryos injected for *dlp* dsRNA, ectopic *wg* transcription is unaffected showing that Dlp is not required for the constitutive activation of Hh signalling. (F) In sibling embryos which are heterozygous for *ptc* and thus wild type for Hh signalling, *dlp* RNAi leads to the disappearance of endogenous *wg* as expected. *ptc* heterozygous and homozygous embryos were separated in this experiment by the presence of *lacZ* expression in the heterozygous embryos (signal at the midline in F).

of the syndecan or glypican families, we have examined the function of the two *Drosophila* homologues of glypicans, *dally* and *dally-like (dlp)*, in the embryonic epidermis. Both glypicans had been suggested previously to play a role in the Wg signalling pathway. Unexpectedly, we found a much more restricted and specific role for the fly glypicans. We have shown by RNAi silencing that Dlp is a segment polarity gene that is absolutely required for Hh signalling. This requirement is specific to the Hh pathway, as we show that RNAi silencing of *dlp* does not affect Wg signalling in embryos. In contrast, RNAi silencing of *dally*, the other homologue of glypicans in *Drosophila*, does not produce a segment polarity phenotype, suggesting that Dally is dispensable for Wg or Hh signalling in embryos. Furthermore, RNAi silencing of both *dally* and *dlp* does not affect Wg signalling, suggesting that they do not function redundantly in this pathway.

Role of Dally-like in the Hedgehog pathway

dlp is a bona fide segment polarity gene since *dlp* RNAi generates embryos that fail to maintain *en* and *wg* expression at mid-embryogenesis, and exhibit a full segment polarity phenotype in the cuticle at the end of embryogenesis (Fig. 1, 2 and not shown). The late disappearance of *en* expression and the single stripe of *rho* expression in *dlp* embryos suggest a loss of Hh activity (Fig. 2). This is confirmed by the fact that when *hh* expression is under heterologous control, ectopic *wg* transcription is lost in *dlp* RNAi embryos, whether Hh is provided autonomously (armGal4 experiments) or non-autonomously (simGal4 experiments) (Fig. 4). These experiments demonstrate unambiguously that *dlp* is required for Hh signalling and rule out a requirement for *hh* transcription.

Dlp is a GPI-anchored protein and is likely to be localised at the cell surface. This leaves two plausible roles for Dlp: either it is required for the release of active Hh from the secreting cells, or it is required for the interpretation of the Hh signal on the receiving cells. Our experiments eliminate several possibilities. First, Dlp is required for the activity of Hh-N, an engineered form of Hh which is pre-processed and unmodified by cholesterol (Fig. 5). This suggests that Dlp is necessary downstream of Hh processing and cholesterol modification. Downstream of these events, Hh undergoes another lipid modification, the addition of a palmitoyl moiety. The segment polarity gene *rasp* codes for an acyltransferase which is thought to be needed for Hh palmitoylation (Amanai and Jiang, 2001; Chamoun et al., 2001; Lee and Treisman, 2001; Micchelli et al., 2002). Thus, Dlp could be required for the function of *rasp* in the signalling cells. However, whereas palmitolylation is essential for Hh-N activity, a recent report shows that it is not strictly required for the activity of wild-type Hh in *Drosophila* embryos (Gallet et al., 2003). This suggests that the cholesterol and palmitoylate modifications might be partially redundant for the activity of wild-type Hh, at least in embryos. Thus, although Dlp could still act at the level of *rasp* on another function, loss of palmitoylation alone cannot account for the complete loss of Hh signalling seen in *dlp* RNAi embryos. It seems therefore more likely that *dlp* functions in the responding cells.

We show that *ptc* is epistatic to *dlp*, indicating that Dlp acts upstream or at the level of the Ptc receptor (Fig. 6D-F). One possibility is that Dlp binds Hh and facilitates its interaction with Ptc. Increasing the concentration of Hh in receiving cells in either armGal4/UAShh or armGal4/UAShh-N experiments, does not abolish the requirement for Dlp. This argues against a role of Dlp in merely increasing the concentration of Hh ligand at the cell surface, and suggests a more specific role. Recent evidence supports a model in which, upon Hh binding, Ptc is endocytosed and inactivated by degradation, and this in turn indirectly activates Smoothened and the Hh intracellular pathway (Denef et al., 2000; Martin et al., 2001; Strutt et al., 2001). Dlp may localise Hh and Ptc in membrane microdomains required for Ptc endocytosis and subsequent degradation.

While we were completing this manuscript, Lum and

colleagues (Lum et al., 2003) reported that Dlp is required for the transduction of the Hh signal in cultured *Drosophila* cells, using RNAi silencing. The design of the experiment eliminates the requirement for Hh secretion or distribution, showing that Dlp is required for the reception of the signal in cultured cells. In agreement with our results, Lum and colleagues find that the requirement for Dlp is suppressed by Ptc RNAi, showing that Dlp acts upstream or at the level of Ptc in their assay (Lum et al., 2003). There are two differences, however, between the in vivo and in vitro data. First, they found that the requirement for Dlp in Hh signal transduction is suppressed by expressing Hh in responding cells. We did not find this in either armGal4/UAShh or armGal4/UAShh-N experiments. This difference may be due to differences in dose: transfection of Hh in cultured cells may generate protein levels well above physiological levels, whereas Gal4 expression levels are in the range of endogenous expression levels. Another difference is that overexpression of Dlp in cultured cells stimulates the response to Hh, in a manner comparable to overexpression of Cubitus interruptus (Ci), the downstream component of the Hh pathway. Using UASdlp and several Gal4 drivers including armGal4VP16, we were not able to detect any stimulation of *wg* transcription in embryos following Dlp overexpression (Fig. 6A). In contrast, overexpression of Ci in embryos does stimulate *wg* transcription (Alexandre et al., 1996) (data not shown). A possibility is that endogenous *dlp* is expressed at low levels in cultured cells and is therefore limiting, but is not limiting in embryos.

Drosophila glypicans and Wingless signalling

We did not find a requirement for the fly glypicans Dlp and Dally in Wg signalling in the embryonic epidermis. In agreement with our findings in vivo, Lum and colleagues showed that RNAi of *dally* or *dlp* does not affect Wg signalling in a cell culture assay (Lum et al., 2003). We found that when Wg expression is under heterologous control (using armGal4 or simGal4), and thus independent of Hh signalling, *en* maintenance and naked cuticle secretion are normal in *dlp* RNAi embryos (Fig. 3). Since these two events are under direct control by Wg in the ventral epidermis, this suggests that Dlp is not necessary for Wg signalling in embryos. RNAi silencing of *dally*, using three different dsRNA sequences and high concentration of dsRNA, did not give any segment polarity phenotype in the cuticle and did not affect the pattern of *en* expression or the secretion of naked cuticle (Table 1, Fig. 1 and 3, not shown). Our RT-PCR experiment demonstrates that *dally* RNAi has worked and leads to a strong reduction in *dally* mRNA levels (Fig. 1E). This result is in contrast with previous reports showing weak segment polarity phenotypes following *dally* RNAi (Lin and Perrimon, 1999; Tsuda et al., 1999; Baeg et al., 2001). It is possible that the use of dsRNA corresponding to larger parts of the *dally* sequence, in these earlier reports, might have resulted in partial silencing of *dlp* through short stretches of sequence homology. To avoid this potential problem, we used short sequences (300-400 nt), and chose sequences with the least homology between *dally* and *dlp* or any other sequences in the genome. Our RT-PCR experiments confirm that RNAi silencing of *dally* did not affect the mRNA levels of *dlp* and vice versa (Fig. 1E).

A possible explanation for an absence of effect in Wg signalling after *dally* or *dlp* RNAi is that the two genes function redundantly in Wg signalling in embryos, as it is the case for the Wg receptors Frizzled and Frizzled2 (Wodarz and Nusse, 1998). We tested this hypothesis by co-injecting *dally* and *dlp* dsRNA in simGal4/UASwg embryos, and show that ectopic Wg signalling is unaffected (Fig. 3F). Importantly, the simGal4 experiments effectively mimic the production of ligand in the wild type because (1) the UAS transgene is expressed in a thin stripe, which allows the monitoring of non-cell autonomous signalling, and (2) the UAS transgene is expressed at levels similar to endogenous transcription (see Fig. 4D). In conclusion, our work suggests strongly that Dally and Dlp, separately or together, are not necessary for Wg signalling in embryos. It has to be noted, however, that if RNAi decreases dramatically the amount of zygotic and maternal mRNAs in embryos, it does not affect the maternal protein stores, and thus we cannot formally rule out that some maternal Dally and/or Dlp protein are sufficient for rescuing Wg signalling in our experiments. Germline clones of null mutations in *dally* and *dlp*, to remove maternal and zygotic contribution of both genes, will need to be performed to definitively settle this issue.

Dally and Dlp could affect Wg distribution or movement without being required for Wg signalling in embryos. We could detect a slight increase in the extent of naked cuticle secreted in enGal4/UASwg [dally RNAi] embryos, which is the assay we use to look at the range of Wg (S.D. and B.S., unpublished). Lum and colleagues also mentioned that Wg distribution at the cell surface is changed in cultured cells silenced for *dally* by RNAi (Lum et al., 2003). This is compatible with an earlier suggestion that HSPGs are needed for the retention of Wg at the surface and within the secretory pathway of expressing cells (Pfeiffer et al., 2002). So removal of Dally might affect Wg distribution in embryos or cell culture, but without detectable impact on Wg signalling. In the wing disc, overexpression of Dlp, but not Dally, has been shown to stabilise Wg at the cell surface (Baeg et al., 2001; Strigini and Cohen, 2000). It has been hypothesised that Notum, which has similarities with pectin acetylesterases, could modify the affinity of Dally and Dlp for Wg (Gerlitz and Basler, 2002; Giraldez et al., 2002). Thus different modifications of the glypicans by tissue-specific enzymes such as Notum, could account for the differences between the embryo and the wing disc. However, it has not yet been proved that there is a direct interaction between Wg and Dally and Dlp, and if this interaction has a biological significance.

Drosophila glypicans and glycosaminoglycan modifications

So far, four genes coding for enzymes necessary for heparan sulphate biosynthesis give a segment polarity phenotype in *Drosophila* embryos: *sugarless* (*sgl*), *sulfateless* (*sfl*), *tout-velu* (*ttv*) and *fringe connection* (*frc*). The segment polarity phenotypes are seen after removal of both maternal and zygotic contribution of any of these genes and are identical to *wg* or *hh* null mutant phenotypes (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999; The et al., 1999; Selva et al., 2001; Goto et al., 2001). *sgl, sfl, ttv* and *frc* have similarities with vertebrate genes coding for a UDP-glucose dehydrogenase, a N-deacetylase/N-sulphotransferase, a heparan sulphate co-polymerase (Ext1), and a UDP sugar transporter, respectively. Consistent with this, mutations in all

four genes affect glycosaminoglycan biosynthesis: Sgl affects both chondroitin and heparan sulphate synthesis, Sfl reduces the proportion of sulphated disaccharides in heparan sulphate, Ttv dramatically decreases all forms of heparan sulphate and Frc reduces the amount of heparan sulphate in embryos (Toyoda et al., 2000; The et al., 1999; Selva et al., 2001). Since glypicans carry heparan sulphate chains, they have been hypothesised to be the target of these enzymes. However, *sfl* and *sgl* have both been implicated in Wg signalling but not Hh signalling in embryos (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999). The fact that neither *dally* nor *dlp* seem to be required for Wg signalling in embryos (our study) or in cultured cells (Lum et al., 2003) poses a paradox. A possibility is that *dally* and *dlp* are solely required for Wg distribution and/or transport, as opposed to Wg signal transduction, and that this requirement is not revealed after RNAi in embryos or in cells. Alternatively, Sfl and Sgl might be required for the function of another heparan sulphate proteoglycan or for the glycosylation of a protein with a central role in Wg signalling. The fourth gene, *ttv*, was found to act specifically in the Hh signalling pathway, and thus could exhibit a segment polarity phenotype because of loss of Dlp activity. However, Ttv is required for Hh movement in wing discs, but not for Hh signalling per se (Bellaiche et al., 1998; The et al., 1999). Dlp is strictly required for Hh signalling but does not seem to influence its movement in embryos, at least in an overexpression assay (Fig. 6A). Furthermore, RNAi silencing of *ttv* and its two *Drosophila* homologues *Ext2* and *Ext3*, individually or in combination, does not inhibit Hh signalling in cell culture (Lum et al., 2003). Thus, the basis for the segment polarity phenotype of *ttv* mutants remains to be clarified.

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