Dally and Notum regulate the switch between low and high level Hedgehog pathway signalling

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SUMMARY

During development, secreted morphogens, such as Hedgehog (Hh), control cell fate and proliferation. Precise sensing of morphogen levels and dynamic cellular responses are required for morphogen-directed morphogenesis, yet the molecular mechanisms responsible are poorly understood. Several recent studies have suggested the involvement of a multi-protein Hh reception complex, and have hinted at an understated complexity in Hh sensing at the cell surface. We show here that the expression of the proteoglycan Dally in Hh-receiving cells in Drosophila is necessary for high but not low level pathway activity, independent of its requirement in Hh-producing cells. We demonstrate that Dally is necessary to sequester Hh at the cell surface and to promote Hh internalisation with its receptor. This internalisation depends on both the activity of the hydrolase Notum and the glycosyl-phosphatidyl-inositol (GPI) moiety of Dally, and indicates a departure from the role of the second glypican Dally-like in Hh signalling. Our data suggest that hydrolysis of the Dally-GPI by Notum provides a switch from low to high level signalling by promoting internalisation of the Hh-Patched ligand-receptor complex.

KEY WORDS: Hedgehog, Morphogen, Proteoglycans, Drosophila

INTRODUCTION

Secreted morphogens are potent signalling factors that often traverse considerable distances to activate their signalling cascades (Rogers and Schier, 2011). Responsive cells must sense and react in a precise and highly dynamic manner to minor changes in morphogen concentration (Dessaud et al., 2008), yet the regulatory mechanisms employed by cells to do this are still a major question in modern biology. The extracellular heparan sulfate proteoglycans (HSPGs) play a major role in this process by regulating morphogen movement and reception in both invertebrates and vertebrates (Gallet, 2011; Lin, 2004; Yan and Lin, 2009). HSPGs consist of a core peptide with branching heparan sulfate (HS) glycos-amino-glycans (GAG) side chains (Bülow and Hobert, 2006; Lin, 2004). A subfamily of HSPGs, the glypicans, which are anchored to the plasma membrane by a glycosyl-phosphatidyl-inositol (GPI), might contribute to the reception of morphogens such as Hedgehog (Hh) (Gallet, 2011). HSPGs are not alone in this role, as other novel proteins have been implicated, including members of the Ihog/Boi (CDON/BOC) families; Ihog and Boi are essential for Hh proteins have been implicated, including members of the Ihog/Boi (CDON/BOC) families; Ihog and Boi are essential for Hh intracellular signalling (Lum et al., 2003; Gallet et al., 2008). Dally contributes to Hh reception and signalling through mediation of Hh-receptor binding by their heparin-like GAG chains. Indeed, in vertebrates the glypican GPC3 competes with PTCH1 for SHH binding, thus inserting a negative influence on signalling (Capurro et al., 2008), whereas GPC5 augments SHH signalling in mammalian systems by increasing SHH binding to PTCH1 through its GAG chains (Li et al., 2011).

During Drosophila development, Hh patterns embryonic and larval tissues such as the developing larval wing imaginal disc where the glypicans Dally-like (Dlp) and Dally regulate Hh pathway activity (Bellaiche et al., 1998; Lum et al., 2003; Han et al., 2004; Takeo et al., 2005; Eugster et al., 2007; Gallet et al., 2008; Ayers et al., 2010; Yan et al., 2010; Williams et al., 2010; Kim et al., 2011). Dlp contributes to internalisation of the Hh-Patched complex (Ptc, a Hh receptor) thus positively regulating Hh intracellular signalling (Lum et al., 2003; Gallet et al., 2008). However, Dlp does not seem to contribute to Hh binding at the cell surface (Yao et al., 2006; Gallet et al., 2008). Dally contributes to long-range spreading of Hh from the secreting cells probably through its direct interaction with the morphogen (Ayers et al., 2010; Eugster et al., 2007; Takeo et al., 2005). Yet a potential role of Dally in receiving cells has not been closely monitored. In addition, GPI anchor cleavage, an event that may allow the release and movement of glypicans in the extracellular space, adds an additional level of complexity that must be taken into account. Indeed, Notum, an enzyme with phospholipase-like activity, is...
thought to cleave glypicans and is implicated in their function in both vertebrates and invertebrates (Ayers et al., 2010; Gerlitz and Basler, 2002; Giráldez et al., 2002; Traister et al., 2008).

We have used the Drosophila wing disc to investigate further the role of the glypican Dally in Hh signalling. We reveal a novel cell-autonomous function for Dally in Hh-receiving cells that respond to the highest level of Hh signal. We show that this action of Dally is mediated through sequestration of Hh, and that Dally’s GPI anchor allows subsequent internalisation of this complex. Moreover, Notum, like Dally, is involved in activation of high-level target genes. We show that Notum operates through Dally to activate high-level target genes, most likely by facilitating Dally internalisation along with Ptc. We propose that Dally sequesters Hh in receiving cells, and subsequent cleavage of Dally’s GPI anchor by Notum enables the co-internalisation of Dally with Hh and its receptor Ptc, to activate the highest level of Hh pathway signalling.

MATERIALS AND METHODS

Fly strain and genetics

UAS-Dally: UAS-GFP::Dally and UAS-GFP::Dally32 were described by Eugster et al. (Eugster et al., 2007), and UAS-Myc::Dally32 and UAS-Dally::TM were described by Takoe et al. (Takoe et al., 2005). dally75F (Franch-Marro et al., 2005) and notumw141 (Gerlitz and Basler, 2002) are null alleles. Loss-of-function clones in the wing imaginal disc were performed as described previously (Xu and Rubin, 1993). UAS-notum was described by Giráldez et al. (Giráldez et al., 2002). Transgenes were overexpressed using the UAS/Gal4 system (Brand and Perrimon, 1993). Overexpression clones in the wing imaginal discs used the ‘flip-out’ technique (Basler and Struhl, 1994) and actin–CD2–Gal4 transgene recombined with a UAS-GFP transgene to mark the clones or the abx–lacZ–gal4 transgene. Clones were induced by heat shocking L1 larvae at 37°C for 10 minutes. Mutant clones of dally or Notum were obtained using FRT-FLP-mediated recombination and heat shocking L1 larvae for 40–60 minutes. Other stocks were obtained from Bloomington Stock Center. All other crosses were maintained at 25°C unless otherwise stated.

Rat anti-Dally antibodies

A Dally peptide (amino acids 450-530) fused to GST was produced in Escherichia coli and purified with a GST affinity column and used to immunise two rats using standard protocols (Eurogentec). We further purified the sera against this Dally peptide using an Affinity-15 column (BioRad). Sera specificity was tested by immunolocalisation on discs and by western blots. One rat antibody gave a weak signal on discs at 1/5 dilution (and showed endogenous staining). Specificity was demonstrated by signal loss in a disc with dally/W; y w loss of function clones (supplementary material Fig. S3A,A’).

Imaginal disc immunostaining, image capture and analysis

Immunostaining was performed as described by Gallet et al. (Gallet et al., 2008). Antibodies were used at the following dilutions: mouse 4D9 monoclonal anti-En [Developmental Studies Hybridoma Bank (DSHB), University of Iowa, IA, USA] at 1/1000 (ascites); rabbit anti-En (Santa Cruz) at 1/1000; mouse N2 7A1 monoclonal anti-β-Catenin (DSHB) at 1/100 (supernatant); mouse 13G8 monoclonal anti-Dllp (DHP) at 1/50 (supernatant); rabbit ‘Calvados’ polyclonal ant-Hh at 1/400 (Gallet et al., 2003); monoclonal anti-Ptc 1/400 (Strutt et al., 2001); polyclonal guinea pig anti-Dally1/200 (Ayers et al., 2010); monoclonal rat anti-Dally 1/5 (see above); monoclonal rabbit anti-βGal at 1/500 (Cappel); monoclonal mouse anti-βGal at 1/500 (Promega); monoclonal chicken anti-βGal at 1/1000 (Gene Tech). Fluorescent secondary antibodies were used at 1/200 for Cy3-conjugated donkey anti-rat, Cy3- or Cy5-conjugated goat anti-mouse, Cy3- or Cy5-conjugated goat anti-rabbit, and donkey Cy3-conjugated anti-chicken (Jackson Laboratory). Fluorescence images were obtained with a Leica Sp DMR TCS-NT confocal microscope and processed using Adobe Photoshop 7.0. Most images are stack projections of four to eight views with 250 nm steps, or single sections at the appropriate level (apical, sub-apical or lateral). z-sections are single sections captured as indicated on the images using the 63× objective and zoom ×2. Plot analyses were carried out using ImageJ software and statistical analyses with Microsoft Excel software.

RESULTS

Daily contributes to high level, but not low level, Hh signalling

In the Drosophila wing imaginal disc, Hh is produced and released from the posterior (P) compartment and diffuses into the anterior (A) compartment, where it is bound by cells that express the Hh reception complex. This triggers an intracellular signalling cascade in response to Hh concentration (Ogden et al., 2004). Close to the source at the anterior-posterior (A-P) boundary, a high level of Hh activates a specific set of transcriptional targets, including the gene engrailed (en). A second set of target genes are turned on in response to medium levels of Hh, including the gene encoding the Hh receptor Ptc. Finally, only low levels of Hh are required to activate a third set of target genes, including decapentaplegic (dpp), which is also repressed by En in the first few rows of A cells.

We have previously shown that the glypican Dlp plays an important positive role in Hh-receiving cells in the wing disc (Gallet et al., 2008). Yet, we have observed that although loss of dlp results in decreased expression of Hh target genes, this is to a lesser extent than in the double mutant for both Drosophila glypicans, dally-dlp (supplementary material Fig. S1A-B’). Indeed in dlp loss-of-function (LOF) clones, En is more weakly affected compared with the double mutant dally-dlp LOF clones (supplementary material Fig. S1A,A’,B,B’). This indicates that Dlp and Daily have complementary roles in Hh-receiving cells. Therefore, we induced dally22 (a null allele) LOF clones in the anterior compartment of the wing imaginal, and observed the effect on Hh target genes. In wild-type (WT) discs, the high level target gene en is expressed in three rows of cells, but in the daily mutant clones this was reduced or lost in an autonomous manner in A cells (Fig. 1A,A’/H11033 compared with 1A’/H11032). To confirm this effect on high level targets, we looked at Smoothened (Smo) staining. Smo is an essential positive member of the Hh pathway (Ayers and Theron, 2010), and is stabilised in response to high levels of Hh in both the P compartment and, to an even greater extent, at the A-P boundary where the Hh pathway is most strongly activated. We found that the A-P stabilisation of Smo was lost in dally mutant clones (supplementary material Fig. S1D,D’), consistent with our observation that high level signalling was reduced. Although high level signalling was affected by Daily loss, we found no effect on the low level target dpp (Fig. 1A,A’c compared with 1A’d; supplementary material Fig. S1C’,C’), using a dpp-lacZ expression reporter. To confirm these observations, we have quantified the effect of Daily loss on En and
dpp expression in numerous clones (Table 1). The results are summarised in Fig. 1D. We conclude that Dally is essential in an autonomous manner for complete high-level Hh pathway signalling in the A cells, whereas Dally is dispensable for low level signalling (Fig. 1E).

**Ectopic Dally in A cells increases the level and domain of expression of high-level Hh target genes**

To better understand the role of Dally in high level signalling, we overexpressed GFP::Dally in clones (using the ‘flipout’ technique, see Materials and methods) in the disc. In accordance with our previous results, we found that ectopic Dally induced an increase in both the expression level and expression domain of En in anterior cells only close to the A-P boundary in a cell-autonomous manner (Fig. 2A,A'/H11033a compared with 2A'/H11033b; Table 1; supplementary material Fig. S2A,A'/H11033a), as if the Dally-dependant activation of the pathway was conditioned to high levels of Hh at the cell surface. Consistent with the well-described activity of En, we observed that in many cases the ectopic En activated by GFP::Dally was strong enough to repress dpp expression (Fig. 2A'/H11033, green arrows; supplementary material Fig. S2A-A'/H11033).

To confirm our results, we expressed Dally in all Hh-receiving cells using the ptcGal4 driver. We found that this caused an increase in the number of cell rows expressing En [four rows of A cells (Fig. 2D,D'/H11033; Table 1)] and, subsequently, a reduction of dpp expression (compare Fig. 2C-C'/H11033 with 2D-D'/H11033). Similar results were obtained with another low level target iroquois (ara, caup, mirr) (supplementary material Fig. S2C-C' compared with S2B-B'). This confirms that Dally plays a role in the induction of high level Hh signalling (En) at the expense of low level targets, such as dpp or iroquois, and is consistent with an autonomous action of Dally on Hh signalling independently of its role in Hh spreading.

**The membrane anchor of Dally is required for Hh stabilisation in Hh-receiving cells**

Next, we wanted to decipher the mechanisms through which Dally contributes to high level Hh signalling. For Dally to be necessary in a cell-autonomous manner in anterior cells at the A-P border it must meet several requirements. First, Dally must be expressed in

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**Table 1. Expression of Hh pathway targets during Dally and Notum misregulation**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mean number of cells expressing En (n)</th>
<th>Mean number of cells expressing dpp (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT dpp-lacZ</td>
<td>2.9 (20)</td>
<td>7.8 (20)</td>
</tr>
<tr>
<td>Anterior dally32 clones</td>
<td>1.8 (14)**</td>
<td>7.9 (18)</td>
</tr>
<tr>
<td>Anterior dally32 clones</td>
<td>4 (20)**</td>
<td>8.1 (15)</td>
</tr>
<tr>
<td>Anterior dally32 clones</td>
<td>2.1 (14)**</td>
<td>14.5 (14)**</td>
</tr>
<tr>
<td>Anterior notumwif141 clones</td>
<td>1.9 (20)**</td>
<td>8.1 (16)</td>
</tr>
<tr>
<td>Anterior notumwif141 clones</td>
<td>3.7 (18)**</td>
<td>7.6 (20)</td>
</tr>
<tr>
<td>Anterior notumwif141 clones</td>
<td>1.3 (20)**</td>
<td>6.6 (12)**</td>
</tr>
<tr>
<td>Anterior notumwif141 clones</td>
<td>3.6 (12)**</td>
<td>18.8 (5)**</td>
</tr>
<tr>
<td>Anterior notumwif141 clones</td>
<td>6 (20)**</td>
<td>13.1 (20)**</td>
</tr>
<tr>
<td>Anterior notumwif141 clones</td>
<td>2.3 (6)*</td>
<td>8.8 (6)**</td>
</tr>
<tr>
<td>Anterior notumwif141 clones</td>
<td>1.8 (18)**</td>
<td>6.4 (18)**</td>
</tr>
</tbody>
</table>

*aP<0.05 compared with WT.

**P<0.001 compared with WT.
these cells. Using a Dally antibody made in-house (see Materials and methods; supplementary material Fig. S3A, A’), we found that Dally is expressed at its highest in the first four to five rows of Hh-receiving cells (Fig. 3A, A’) as well as showing expression along the dorsoventral (D-V) axis and at the extremities of the disc. The A-P expression is consistent with dally transcript levels (Fujise et al., 2001; Tsuda et al., 2001), and with the observed requirement of Dally on high-level Hh targets (Fig. 1). It is interesting, however, to note that Dally is expressed in cells that respond to low level signalling, i.e. those that express dpp. It has been suggested that Dally plays a role in Dpp signalling (Fujise et al., 2003; Takeo et al., 2005), which may explain the requirement for Dally expression in these cells, i.e. to ensure that Dpp signalling is not affected even when Hh signalling is.

The second requirement is that Dally must have an instructive role in Hh signalling in receiving cells independent of its role in Hh spreading from posterior cells. Because Dally positively regulates Hh accumulation levels in the Hh-producing cells (Ayers et al., 2010; Eugster et al., 2007), we investigated whether Dally behaves similarly in the Hh-receiving cells. We observed that ectopic Dally did increase the levels of visible Hh at the apical surface in the A cells (Fig. 3B-C’). As glypcans are known to be dynamic proteins (subject to cleavage, release or even active internalisation) (Capurro et al., 2008; Eugster et al., 2007; Gallet et al., 2008; Traister et al., 2008), we employed various transgenic constructs for Dally to determine which forms of Dally (supplementary material Fig. S3B) could cause Hh accumulation. Interestingly, we found that Hh was accumulated independently of Dally’s GPI anchor, as a form of Dally in which the GPI anchor is replaced with a transmembrane domain (Dally::TM) (Takeo et al., 2005) still caused increased levels of Hh on the apical surface of receiving cells (Fig. 3D-E’). Then we used a secreted form of Dally (DallySec) that is released without being anchored to the plasma membrane (Eugster et al., 2007; Takeo et al., 2005) and found that Hh is not accumulated at the apical surface of DallySec-expressing cells (supplementary material Fig. S3C-D’, inset). Taken together, we conclude that Dally promotion of Hh levels at the apical surface of the wing disc epithelium requires linkage to the plasma membrane (by either its native GPI anchor or another TM anchor), and is seen in the Hh-receiving cells independently of Dally’s role in the P cells.

Another requirement for a defined role of Dally in receiving cell signalling is demonstration that the action of Dally is independent of the second Drosophila glypcan Dlp. This is important because we know that Dlp is also necessary for high level Hh signalling (Gallet et al., 2008). To address this issue of functional redundancy between Dally and Dlp we attempted rescue experiments. Rescue of dlp−/−...
lethality at larval stage 2-3 by expression of Dally in the receiving cells was not successful in our hands, and in fact often caused greater lethality at an earlier stage (data not shown), supporting the idea that these two glypicans are not functionally redundant. In addition, no significant changes in Dlp expression were observed in Dally mutant clones (supplementary material Fig. S3E-G'), indicating that the protein levels of Dlp and Dally are independently regulated. However, it is still possible that Dally is acting through Dlp to cause Hh sequestration. If this were the case, one would expect that overexpression of Dlp or Dlp::CD2 (a transmembrane form of Dlp highly localised to the membrane) (Gallet et al., 2008) would also cause Hh accumulation. We found that Dlp::CD2 expression had no effect of Hh levels at the plasma membrane (PM), whereas Dlp overexpression caused a decrease in Hh levels (data not shown; Gallet et al., 2008). Therefore, we conclude that Dally is acting in a Dlp-independent manner to sequester Hh in receiving cells, where it acts to promote high level intracellular signalling.

Notum regulation of Dally is required for high level Hh target gene expression

As we have found that Dally positively regulates Hh levels at the apical surface irrespective of its membrane anchor, we wanted to address whether Dally::TM can also cause ectopic high level Hh signalling. To this end, we overexpressed Dally::TM in clones or using the ptc-Gal4 driver, in the same conditions that were used for GFP::Dally. Intriguingly, we discovered that this form of Dally was able to ectopically activate low level Hh target genes, such as dpp (Fig. 4A,A'; data not shown); however, no change in En expression was observed (Fig. 4A,A'; data not shown) and even a weak reduction in the expression of En-expressing cells compared with WT was seen (Table 1). Thus, the GPI anchor of Dally is essential for ectopic activation of high level Hh signalling, although transmembrane-tethered Dally can activate low level signalling, perhaps owing to its ability to sequester Hh. From this, we suggest that Dally has two roles: it is involved first in aiding apical Hh accumulation which may be sufficient to activate low level signalling in cells close to the A-P boundary, and then in a second event that depends on Dally’s GPI anchor and regulates the switch to high level Hh signalling. As activation of high level targets by Dally requires the presence of a GPI anchor, we next investigated what the role of this GPI anchor could be. In vertebrates, the GPI anchors of glypicans are subject to cleavage by the extracellular enzyme Notum (Traister et al., 2008), and we have previously shown that Notum augments Hh spreading through its regulation of Dally (Ayers et al., 2010). We were therefore interested in whether the Notum enzyme has a role in the Hh-receiving cells, and whether this is specific to high level Hh signalling.

We first analysed whether removing Notum activity in A cells would affect Hh target gene expression. A decrease in En expression similar to that seen in dally mutant clones was observed (Fig. 4B-B'; Table 1; supplementary material Fig. S4). Intriguingly, this is different to what is seen when Notum is removed in just the P compartment (Ayers et al., 2010), indicating that Notum has differential activities in different compartments. We then overexpressed Notum using ptc-Gal4. Analogous to Dally overexpression, we found an increased domain of cells expressing En, which in turn represses dpp expression posteriorly (Fig. 4C-C'; Table 1) compared with WT (Fig. 2C-C'; Table 1).

To demonstrate definitively that Notum activity targets Dally protein, we overexpressed Notum in a dally mutant background, reasoning that if Notum acts on Hh pathway signalling through its regulation of Dally, removing Dally should alleviate the Notum overexpression phenotype. Indeed, in the absence of Dally, Notum overexpression was unable to induce ectopic En (Fig. 4D-D'; Table 1). Moreover, dpp expression was also reduced to a similar extent as that we had already observed in dally mutant animals (Ayers et al., 2010), probably owing to the well-accepted role of Dally in Hh spreading from the posterior cells.
Notum acts on the GPI anchor of Dally to contribute to high level pathway signalling through several mechanisms

Our data indicate that in Hh-receiving cells Notum acts through its action on Dally to regulate high level intracellular Hh signalling. We wondered then whether in these A cells, the GPI anchor of Dally could be a substrate for Notum, and postulated that in the absence of endogenous Dally, Notum should not be able to activate En through Dally::TM. To test this hypothesis, we expressed Notum and Dally::TM in Hh-receiving cells in a dally null mutant background. No ectopic En expression was observed; indeed, the contrary was seen with En expression similar to anterior dally null mutant clones (Fig. 5A,A/H11032; Table 1). Moreover, dpp expression was also reduced (Fig. 5A,A/H11033; Table 1) in a manner comparable to what we have previously observed in a dally null background (Ayers et al., 2010). Therefore, to maintain high level signalling in the anterior cells, there is a requirement for both Notum and GPI anchored-Dally. This is supported by the fact that the co-expression of Dally::TM and Notum (in a WT disc) abolishes the wider En expression observed when Notum is overexpressed alone (compare Fig. 5B and 5B/H11032; Fig. 4C,C’). We suggest that this is caused by Dally::TM titrating Notum from endogenous Dally, thus acting as a dominant negative to inhibit endogenous Dally cleavage and signalling. We also noted that in this case dpp expression is wider (Fig. 5B,H11033; Table 1) than WT, consistent with our previous observation that Dally::TM causes ectopic low level signalling.

Our data have led us to conclude that Dally modification by Notum activates high level signalling. Yet it was not clear what the molecular basis of this event was. If Dally cleavage and release into the extracellular space was the defining factor in pathway activation, then overexpression of a form of Dally missing the GPI (DallySec; supplementary material Fig. S3B), that is secreted directly, should mimic overexpression of Dally and Notum together. However, whereas DallySec expressed with ptcGal4 caused ectopic dpp in a wide domain (Fig. 5C,C/H11033; Table 1), we only observed a very slight increase in the En expression domain (Fig. 5C,C/H11033; Table 1). Yet when both Dally and Notum were overexpressed, the En expression domain was far broader compared with single Notum, Dally or DallySec overexpression (Fig. 5D,D/H11033; compare Fig. 5D,D/H11033 compared with Fig. 5D,D/H11033; Fig. 5C,C’; Table 1). Strikingly, dpp expression was also broader (Fig. 5D,D/H11033; Table 1), and shifted anteriorly owing to repression by a wider En expression. Thus, whereas Dally/Notum co-expression augments both low and high level signalling, DallySec expression only causes a large increase in low level targets. The major difference in these cases is the presence of a GPI anchored-Dally in the former, and it therefore appears that anchoring Dally to the PM limits Dally
activity to cells with a high level of Hh signalling. We hypothesise that, unlike constitutively secreted Dally, GPI-Dally (WT Dally) might require initial localisation to the PM to bind other important members of the pathway, such as receptors.

Dally regulates Ptc trafficking

To investigate further the role of Dally in Hh reception we followed the behaviour of the Ptc receptor in the absence of Dally or Notum. In Dally LOF clones in the anterior we found that although Ptc expression (number of cells expressing Ptc) was not affected (Fig. 6A,A/H11033, B,E), the size, number and intensity of Ptc vesicles were reduced (Fig. 6A-A/H11033, C,E, compare 6A/H11033c with 6A/H11033d; supplementary material Fig. S1C,C/H11033). Consistent with these observations, we also found that ectopic expression of GFP::Dally caused an increase in both Ptc vesicle size and number (Fig. 6F-F/H11630; see below). Given these results, we postulated that Dally contributes to Ptc vesicle localisation, perhaps through promoting its interaction with Hh and, thus, its internalisation. We also observed that in Notum mutant cells Ptc vesicles intensity was also reduced (supplementary material Fig. S4). This effect was very strong, probably owing to the large clone size we produced to counteract the non-autonomous action of Notum. Thus, Notum and Dally both contribute to Ptc vesicular localisation. Taken together, our data suggest that the efficiency of GPI Dally in activating high level target genes relies on both its action on Ptc and its cleavage by Notum. Thus, it is conceivable that Notum cleavage of Dally is required for Dally to promote Ptc internalisation (or vice versa) and thus signalling.

Dally is internalised with the reception complex, an event that is promoted by Notum

To investigate this in the imaginal disc cells, we used flip-out clone experiments to create clones of GFP::Dally-expressing cells in both the anterior and posterior compartment. GFP::Dally in the posterior cells is found mostly at the PM, with occasional puncta observed, usually less than one punctate per cell (Fig. 7A/A/H11033c). These puncta are always associated with cytoskeleton (actin), and represent internalised structures as they colocalise with FM4-64, an endocytic marker (data not shown). In the anterior, GFP::Dally is still present at the PM; however, many more Dally vesicles were observed (Fig. 7A-A/H11033c), and a notable discrepancy between anterior and posterior cells is the absence of...
Ptc in the latter. In addition, GFP::Dally expression increased the size of Ptc internal puncta (Fig. 7A', compare a' with WT b'), thus suggesting that in the presence of Ptc, Dally is internalised and might in turn augment Ptc endocytosis and/or accumulation in endosomes. As Dally is probably cleaved by Notum in these cells, it is likely that a large percentage of GPI-cleaved GFP::Dally is present in Ptc-positive internal vesicles, and not secreted from the cell. To test this, we expressed Notum and GFP::Dally using ptcGal4 (Fig. 7C) and found that Notum enhanced the punctuated staining of GFP::Dally (which colocalised with Ptc) (Fig. 7C compared with 7B and 7E). This implies that Dally modification by Notum increases the levels of intracellular Dally puncta, in a Ptc-dependent manner in vivo. In agreement, we observed that when Ptc is also overexpressed with GFP::Dally, increased intracellular GFP::Dally is seen at the expense of apical GFP::Dally, which is depleted (Fig. 7G-G' compared with 7F-F'). Finally, co-overexpression of Ptc and GFP::Dally in the posterior cells brought the level of Dally puncta to equal that observed in the anterior cells (supplementary material Fig. S5A-A').

To show definitively a requirement for Ptc in Dally internalisation required us to observe Dally in the absence of Ptc. This analysis in the receiving cells was hindered by lethality of ptc null alleles in homozygous context. Moreover, we were unable to build a recombinant chromosome bearing both a ptc null allele in combination with specific anterior drivers (such as ptc-Gal4). In addition, observing endogenous Notum directly in vivo is not currently possible, so we turned to cell culture experiments. Using the information provided by the Drosophila RNAi Screening Center (http://www.flyrnai.org/cgi-bin/RNAi_expression_levels.pl) (supplementary material Fig. S6A), we chose to use two Drosophila cell culture lines. First, Clone 8 (Cl8) cells because there is little to no expression of endogenous Notum, allowing us to control the levels through transfection. Second, we used S2R+ cells, which already express a low level of ptc, allowing us to investigate the role of Ptc in Notum and Dally function.

In transfected Cl8 cells, DallyFlag mostly localised to the PM and to a few vesicles (average of 1.2 per cell; Fig. 8G, I). These did not colocalise with the Golgi markers GMAP (Friggi-Grelin et al.,...
or Manosidase::GFP (Bard et al., 2006) and are probably of endocytic nature (Fig. 8A; data not shown). Intriguingly, when Notum-HA was co-transfected with DallyFlag, we observed a fourfold increase in Dally-containing puncta, and many of these also contained Notum (Fig. 8A-a-a', b-c, F-F'); Clones overexpressing GFP::Dally. (B) ptc-Gal4>GFP::Dally. (C) ptc-Gal4>Notum; GFP::Dally. (D) Graph of GFP::Dally vesicle numbers in the A compartment (ptc-Gal4 driver) versus the P compartment (hh-Gal4 driver). (E) Graph of the number of Ptc/GFP::Dally-containing vesicles in the A compartment (ptc-Gal4 driver) overexpressing GFP::Dally alone (B) or both Notum and GFP::Dally (C). (G-G') Clones overexpressing both GFP::Dally and Ptc. F-G' are xz confocal views with apical (Ap) up. Bl, basolateral. Wing discs are labelled for Dally (green), Ptc (red) and Hh (blue). GFP::Dally overexpression in the A compartment increased Ptc vesicles size (compare a' with b). The co-overexpression of Notum and GFP::Dally in Hh-receiving cells increased the number of Ptc/GFP::Dally vesicles (C, E) compared with overexpression of GFP::Dally alone (B, E). Overexpression of Ptc reduces the apical localisation of GFP::Dally, instead favouring its internalisation (compare F-F' with G-G', and arrows in F and G'). Wing imaginal discs are oriented with anterior to the left and dorsal down (except F-G'); white dotted lines mark A-P boundaries. n, number of clones or discs analysed. Error bars represent s.e.m.

DISCUSSION

The function of Dally and, hence, the glypicans in Hh-receiving cells

We have found that, similar to its orthologue Dlp (Gallet et al., 2008), the glypican Dally plays a role in Hh-receiving cells. Yet, although Dally and Dlp have structural and functional similarities
(Bülow and Hobert, 2006), these proteins display important differences that have led us to conclude that they have distinct roles. At the cellular level, Dally is located at the plasma membrane with some internal puncta, whereas Dlp is mostly located within intracellular vesicles and is rapidly endocytosed from the apical surface (Gallet et al., 2008). Membrane-tethered Dally (e.g. by its GPI anchor or a transmembrane domain) consistently augments Hh levels at the cell surface, whereas Dlp cannot, even if constitutively anchored to the plasma membrane (Gallet et al., 2008). Finally, whereas Dally appears to be a substrate for Notum (this study) (Ayers et al., 2010; Han et al., 2005), Dlp does not (Gallet et al., 2008; Han et al., 2005). All of these points are underlined by the fact that rescue experiments (in which Dally was expressed in a Dlp-null background) were never successful in our hands (unpublished data). In addition, Dally loss has no effect on Dlp levels or localisation (supplementary material Fig. S3F,G). These data indicate that these proteins have unique roles during Hh pathway signalling in the receiving cells, echoing what we have previously observed in the posterior/secreting cells (Ayers et al., 2010; Callejo et al., 2011; Gallet et al., 2008).

In light of this, how do we consolidate the functions of Dally and Dlp in Hh-receiving cells? We postulate that in cells close to the Hh source (indeed the domain of Dally expression), apical Dally helps to trap Hh at the cell surface (Fig. 8H). Such a local increase of Hh concentration at the cell surface would promote or facilitate Hh interaction with its reception complex (including Ptc and Dlp) (Gallet, 2011). Dlp is then involved in the rapid endocytosis of the complex Dally-Hh-Ptc (Gallet et al., 2008), a process aided by...
Dally-GPI anchor cleavage by the Notum protein, and resulting in sustained activation of high level intracellular Hh pathway signalling (Fig. 8H).

The molecular function of Notum and its versatile role in Hedgehog signalling

The data presented here and in our previous paper (Ayers et al., 2010) suggest that Notum plays important yet distinct roles in Hh-secreting and -receiving cells. We have found that in the Hh-secreting cells, Notum promotes Dally sheding into the extracellular space, and this is essential for proper long-range spreading of Hh to receiving cells to activate low level long range signalling (Fig. 8I) (Ayers et al., 2010; Eugster et al., 2007). Furthermore, in these cells a form of Dally that lacks the GPI anchor (Dally$^{sec}$) behaves like Notum-processed Dally (Ayers et al., 2010; Eugster et al., 2007; Takeo et al., 2005). However, our study suggests that, contrary to its role in the posterior cells, Notum plays a more autonomous role in the Hh-receiving cells. Indeed, Notum modification of Dally in these cells appears to lead to a local activation of high level signalling (Fig. 8H). In western blot analysis of larval imaginal disc extracts from animals overexpressing either GFP::Dally or both GFP::Dally and Notum using ptcGal4, we found that Notum overexpression led to an increased level of Dally protein (supplementary material Fig. S6B). This is consistent with our observation in discs where Notum increases intracellular levels of Dally in Hh-receiving cells. Intriguingly, Ptc seems to be key in controlling the commitment of Notum-regulated Dally in one or other of these fates. In the absence of Ptc (i.e. in Hh-producing cells), Notum provokes the release of Dally in the extracellular space, whereas in receiving cells Ptc is upregulated in the same domain as Dally and appears to induce internalisation of Dally, rather than release (Fig. 8HJ). Thus, Notum regulates Hh pathway signalling autonomously in receiving cells by facilitating Dally internalisation with Ptc and Hh. We find it intriguing that this conserved relationship between Notum, Dally and the Hh pathway has been harnessed to provide unique signalling mechanisms in different cells. Yet, by acting in conjunction, all of these functions are required for proper long range/low level and short range/high level signalling of the Hh pathway, and thus correct morphogenesis of the wing imaginal disc.

The exact enzymatic activity of Notum is still unknown. Indeed, although Notum presents some similarities to α/β-hydrolases (Giráldez et al., 2002), which are known to modify pectins (GAG-like chains) in plants, evidence suggests that Notum has a phospholipase-C-like activity in vertebrates to cleave glypican GPI anchors (Traister et al., 2008). Although the exact nature of Notum modification of Dally in vivo is not clear, both of our studies suggest a link between the presence of a GPI anchor of Dally and Notum activity, and thus strongly suggest that GPI hydrolysis is the key modification performed by Notum. However, we must note that we cannot rule out the possibility that Notum modifies Dally in additional ways; for example, it could change Dally’s affinity for the receptor complex or its plasma membrane localisation [by relocating Dally to the lipid rafts (e.g. Gallet, 2011)]. Our cell culture data has provided important information about the localisation of Notum in cells that express Dally and Ptc, information that has not been previously available. Notum was most often present at the PM and, in some instances, was also seen in intracellular puncta, both subcellular localisations that also contained Dally, suggesting that a direct interaction between these proteins is essential for their role in Hh pathway signalling.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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