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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 signalling in flies (Yao et al., 2006; Camp et al., 2010; Zheng et al., 2010) and BOC and CDON are essential Shh co-receptors in mammals (McLellan et al., 2008; Kavran et al., 2010; Allen et al., 2011; Izzi et al., 2011). Interestingly, these proteins interact with the extracellular matrix component heparin, which is required for the dimerisation of Ihog and its high-affinity interaction with Hh (McLellan et al., 2006). Hh proteins are also known to interact with heparin and HSPGs through two distinct domains, and loss of this interaction reduces Hh signalling potency (Chang et al., 2011). In the mouse cerebellum, mutation of an HSPG-interacting site in SHH decreases SHH localisation to the proliferating zone and dramatically alters the transcriptional response in these cells (Chan et al., 2009). It is therefore conceivable that GPI-linked glypicans contribute to Hh reception and signalling though 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

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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::Dally^{Sec}* were described by Eugster et al. (Eugster et al., 2007), and *UAS-Myc::Dally^{Sec}* and *UAS-Dally::TM* were described by Takeo et al. (Takeo et al., 2005). *dally^{32}* (Franch-Marro et al., 2005) and *notum^{wf141}* (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^{32}* 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., 2006). 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-Dlp (DSHB) at 1/50 (supernatant); rabbit 'Calvados' polyclonal anti-Hh at 1/400 (Gallet et al., 2003); monoclonal anti-Ptc 1/400 (Strutt et al., 2001); polyclonal guinea pig anti-Dally 1/200 (Ayers et al., 2010); polyclonal 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.

Cell culture

Transfections for immunostaining were carried out as follows: 2×10^5 cells/chamber (Cl8 or S2R+) were seeded on 4-chamber slides and left to recover for 4 hours. Plasmid DNA (500 ng) was used for transfection with Cellfectin (Invitrogen). After transfection, cells were grown over 48 hours before processing for immunostaining. pUAS-Flag-Dally was provided by J. P. Vincent (NIMR, London, UK) and the pUAS-Notum vector by S. Cohen (A*STAR, Singapore). Notum cDNA was cloned from this vector into pPAC using *Eco*R1-*Xba*1 sites in frame with three HA tags to make pPAC-Notum-HA. Guinea pig anti-Dally (Ayers et al., 2010) was diluted to 1/400. Mouse anti-HA (Sigma) was used at 3 μl/10 ml.

RESULTS

Dally 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″) (Gallet et al., 2008; Han et al., 2004). 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 Dally have complementary roles in Hh-receiving cells. Therefore, we induced *dally^{32}* (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 *dally* mutant clones this was reduced or lost in an autonomous manner in A cells (Fig. 1A,A′*a* compared with 1A′*b*,B; e.g. Fig. 6A,A′*a* compared with 6A′*b*,D; supplementary material Fig. S3G,G″; Table 1). 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 Therond, 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 Dally loss, we found no effect on the low level target *dpp* (Fig. 1A,A″*c* compared with 1A″*d*,C; supplementary material Fig. S1C,C′), using a *dpp-lacZ* expression reporter. To confirm these observations, we have quantified the effect of Dally loss on En and

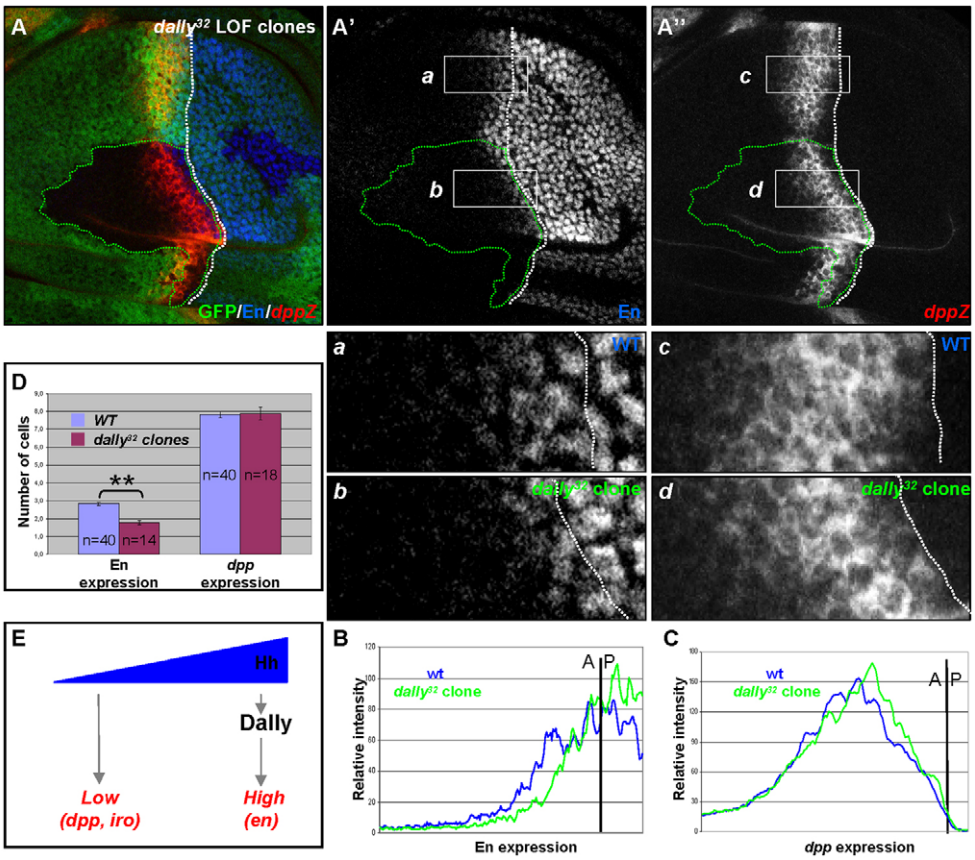

Fig. 1. Dally contributes to high level, but not low level, Hh signalling in *Drosophila*. (**A-A″*d***) Wing imaginal disc with *dally*^{32} LOF clones. Mutant clone is visualised by the lack of GFP. Blue, En expression; red, *dpp-lacZ* (*dppZ*) expression. (a,c) Wild-type tissue; (b,d) *dally* LOF clones. (**B**,**C**) Graphical representation of En (B) and *dpp* (C) expression inside or outside of the mutant clone of the disc shown in A. (**D**) Graph representing the mean number of En- and *dpp*-expressing cells in *dally*^{32} LOF clones compared with WT. (**E**) Schematic of Hh gradient (blue) and the expression of target genes (red) with the potential role of Dally. Wing imaginal discs are oriented with anterior to the left and dorsal down; green dotted lines delineate the mutant clones and white dotted lines mark A-P boundaries. n, number of clones or discs analysed. Error bars represent s.e.m.

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′*a* compared with 2A′*b*,B; Table 1; supplementary material Fig. S2A,A′*a*), 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′,A″, green arrows; supplementary material Fig. S2A-A″*a*). 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′; Table 1) instead of the three rows observed in WT (Fig. 2C,C′; Table 1)] and, subsequently, a reduction of *dpp* expression (compare Fig. 2C-C″ with 2D-D″). 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

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

Genotype	Mean number of cells expressing En (*n*)	Mean number of cells expressing *dpp* (*n*)
WT *dpp-lacZ*	2.9 (20)	7.8 (20)
Anterior *dally*^{32} clones	1.8 (14)**	7.9 (18)
ptc>GFP::Dally (25°C)	4 (20)**	8.1 (15)
ptc>TM::Dally (25°C)	2.1 (14)**	14.5 (14)**
Anterior *notum*^{wif141} clones	1.9 (20)**	8.1 (16)
ptc>Notum (25°C)	3.7 (18)**	7.6 (20)
ptc>Notum; dally^{32} (25°C)	1.3 (20)**	6.6 (12)**
ptc>sec-Dally	3.6 (12)**	18.8 (5)*
ptc>Notum; GFP::Dally (18°C)	6 (20)**	13.1 (20)**
ptc>Notum; TM::Dally (25°C)	2.3 (6)*	8.8 (6)*
ptc>Notum; TM::Dally, dally^{32}	1.8 (18)**	6.4 (18)**

*P<0.05 compared with WT.
**P<0.001 compared with WT.

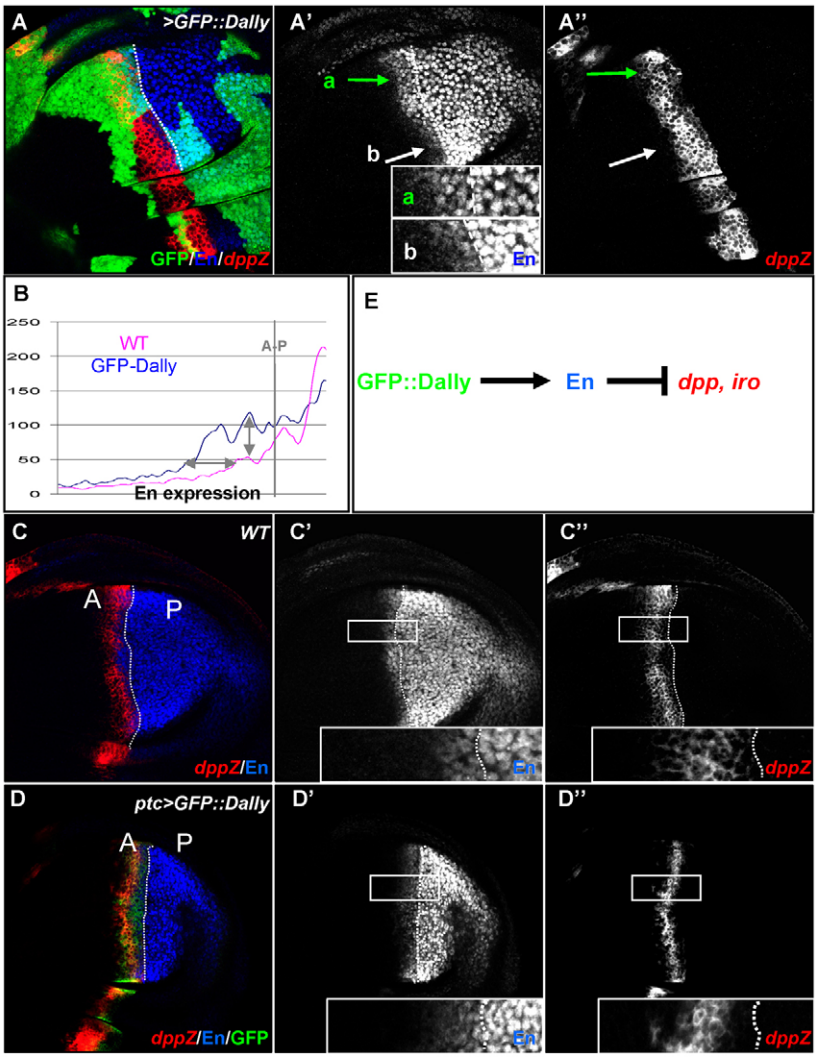

Fig. 2. Ectopic Dally in Anterior cells increases the level and domain of expression of high level Hh target genes. (**A-A″**) Overexpression of GFP::Dally in clones (green) at 29°C. Green arrows indicate the overexpression clone and white arrows indicate the surrounding wild-type tissue. (**B**) Graph representing En expression in a GFP::Dally-overexpressing clone (a in A′) compared with WT tissue (b in A′). (**C-D″**) WT (C-C″) and *ptc-Gal4*>GFP::Dally (green) (D-D″) discs. Blue, En; red, *dppZ*. (**E**) Scheme showing that the overexpression of GFP::Dally increases the expression of En, in turn repressing the expression of *dpp*. Wing imaginal discs are oriented with anterior to the left and dorsal down. White dotted lines mark A-P boundaries.

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 glypicans 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 anchors 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 (Dally^{sec}) 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 Dally^{Sec}-expressing cells (supplementary material Fig. S3C-D′) but, conversely, can be observed colocalising with Dally^{Sec} far from the overexpressing domain (supplementary material Fig. S3C, 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* glypican 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*$^{-/-}$

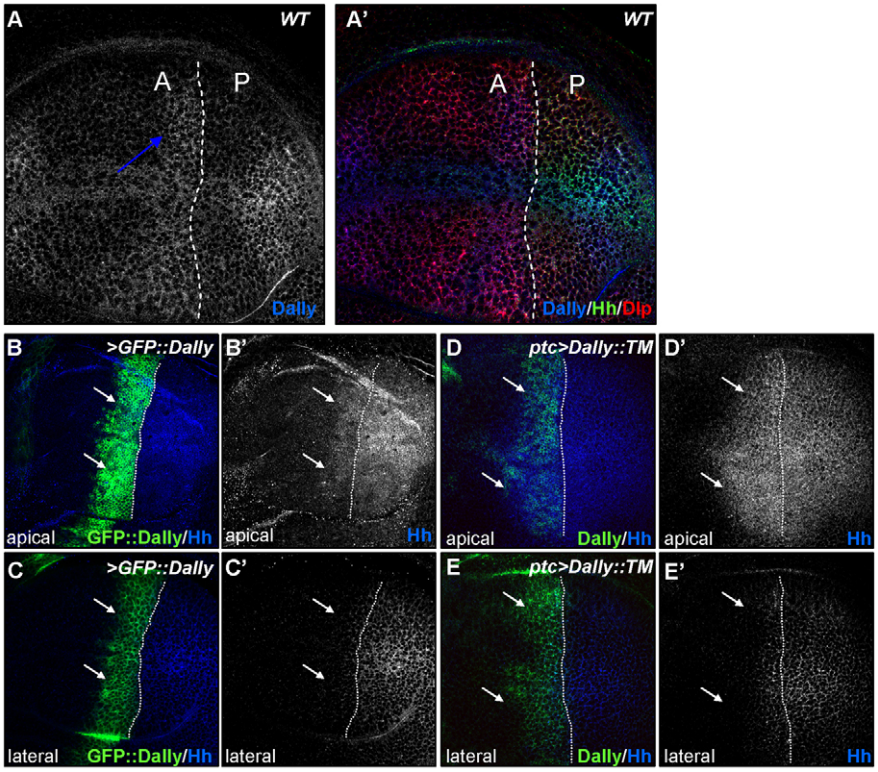

Fig. 3. The membrane anchor of Dally is required for Hh stabilisation in Hh-receiving cells. (**A**,**A′**) WT disc stained for Dally (blue), Dlp (red) and Hh (green). Dally is found at higher levels along the A-P boundary (blue arrow in A). (**B-C′**) GFP::Dally expressed with the *ptc-Gal4* driver (green). GFP::Dally overexpression increases the levels of apical Hh (blue) to a greater extent than lateral Hh (compare arrows in B,B′ with those in C,C′). (**D-E′**) Dally::TM expressed with the *ptc-Gal4* driver (green). Dally::TM also leads to apical (arrows in D,D′), but not lateral (E,E′) accumulation of Hh. Wing imaginal discs are oriented with anterior to the left and dorsal down. White dotted lines mark A-P boundaries.

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 either in clones or using the *ptcGal4* 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 number 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 *ptcGal4*. 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.

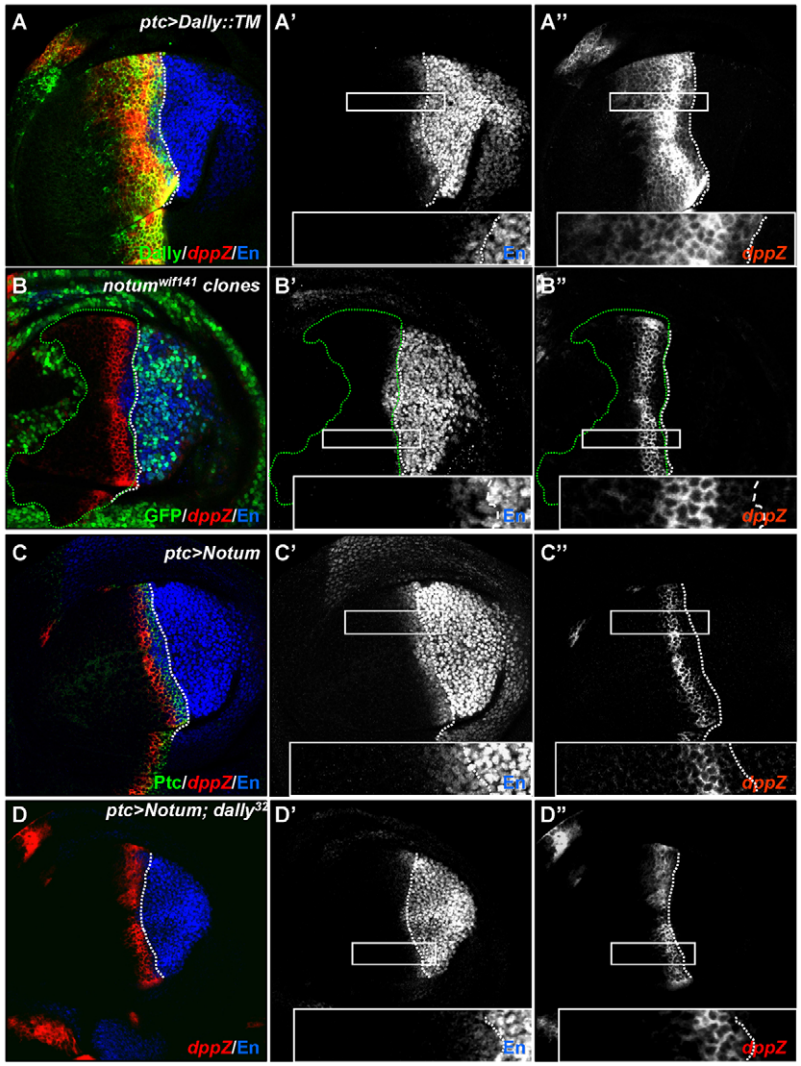

Fig. 4. Notum regulation of Dally is required for high level Hh target gene expression. (**A-D″**) ptc-*Gal4*>Dally::TM (A-A″) and >Notum (C-D″) in WT (A-A″,C-C″) or *dally*^{32} (D-D″) background. (B-B″) *notum*^{wif141} LOF clones (marked by the absence of GFP). Blue, En; red, *dppZ*; green, Dally::TM (A), GFP (B) or Ptc (C). Wing imaginal discs are oriented with anterior to the left and dorsal down; green dotted lines delineate the mutant clones and white dotted lines mark A-P boundaries.

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′; Table 1). Moreover, *dpp* expression was also reduced (Fig. 5A,A″; 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′; 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″; 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 (Dally^{Sec}; supplementary material Fig. S3B), that is secreted directly, should mimic overexpression of Dally and Notum together. However, whereas Dally^{Sec} expressed with *ptcGal4* caused ectopic *dpp* in a wide domain (Fig. 5C,C″; Table 1), we only observed a very slight increase in the En expression domain (Fig. 5C,C′; Table 1). Yet when both Dally and Notum were overexpressed, the En expression domain was far broader compared with single Notum, Dally or Dally^{Sec} overexpression (Fig. 5D,D′ compared with Fig. 2D′, Fig. 4C′ and Fig. 5C′; Table 1). Strikingly, *dpp* expression was also broader (Fig. 5D,D″; 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, Dally^{Sec} 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

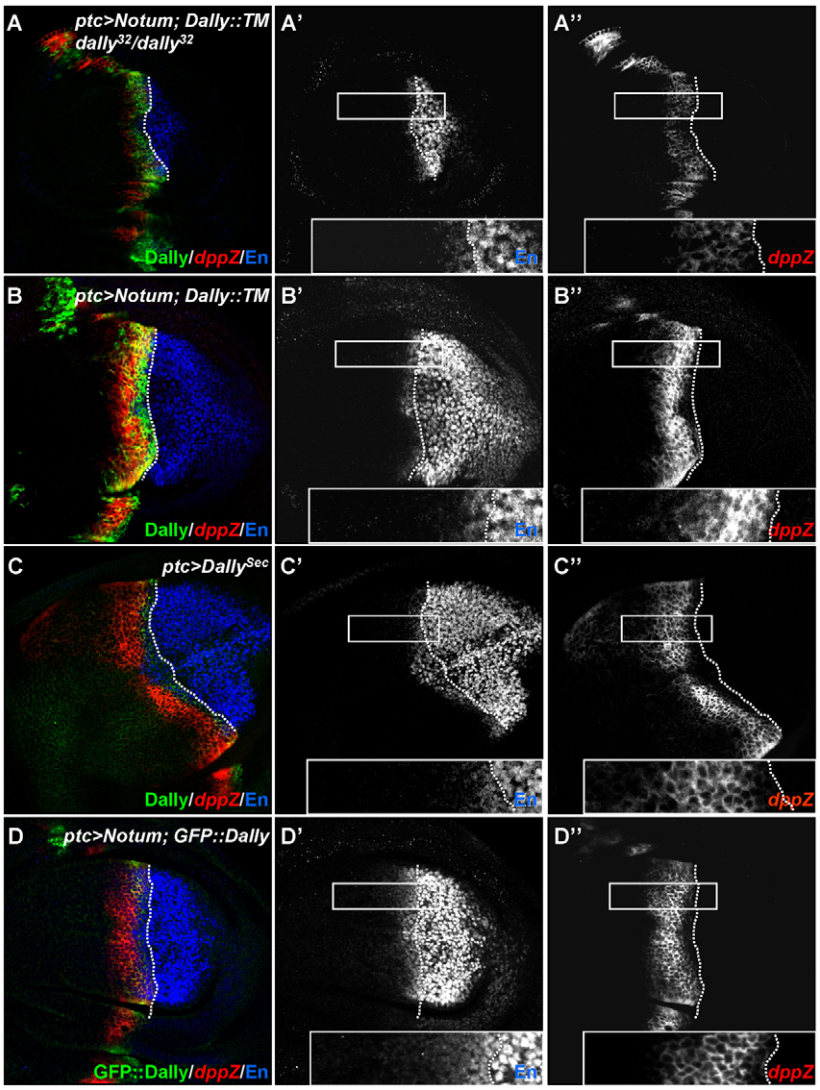

Fig. 5. Notum acts on the GPI anchor of Dally to contribute to high level pathway signalling through several mechanisms. (**A-D″**) *ptc-Gal4>Notum; >Dally::TM, dally*$^{32/32}$ (A-A″), *ptc-Gal4>Notum; >Dally::TM* (B-B″), *ptcGal4>Dally*^{Sec} (C-C″) and *ptc-Gal4>Notum; >GFP::Dally* (D-D″) imaginal wing discs stained for En (blue) and *dppZ* (red). Dally constructs are visualised in green. In the absence of *dally*, the overexpression of both Notum and Dally::TM does not rescue En or *dpp* expression (A-A″). Overexpression of Dally::TM mitigates increases in En expression induced by Notum overexpression, whereas *dpp* is enlarged (B-B″). Dally^{Sec} overexpression promotes a mild En and a strong *dpp* expansion (C-C″) whereas overexpression of both Notum and GFP-Dally results in a wide increase of En expression and a moderate expansion of *dpp* expression (D-D″). Wing imaginal discs are oriented with anterior to the left and dorsal down. White dotted lines mark A-P boundaries.

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″,B,E), the size, number and intensity of Ptc vesicles were reduced (Fig. 6A-A″,C,E, compare 6A″*c* with 6A″*d*; supplementary material Fig. S1C,C″). 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‴; 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 punctuate per cell (Fig. 7A,A″*c*). 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″, 7A″*a*‴ compared with 7A″*c*). A similar discrepancy between anterior and posterior GFP::Dally was observed using *ptc*Gal4 and *hh*Gal4 drivers (Fig. 7D).

We investigated why Dally might be more readily internalised in Hh-receiving cells. In these cells, GFP::Dally puncta often colocalise with both Ptc and Hh (Fig. 7A-A″*a*‴), and a notable difference between anterior and posterior cells is the absence of

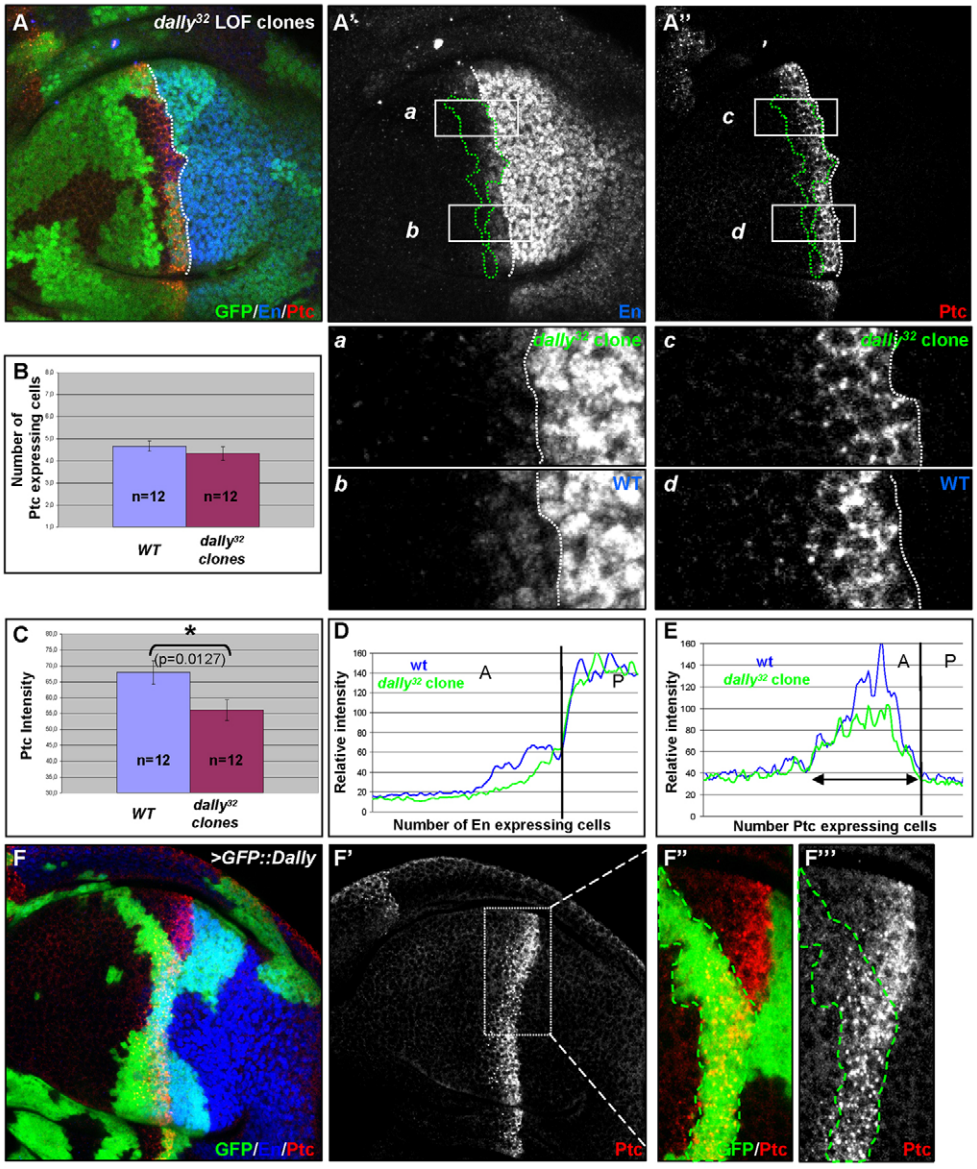

Fig. 6. Dally regulates Ptc trafficking. (**A-A″*d***) Wing discs bearing *dally*^{32} LOF clones (marked by the lack of GFP) labelled for En (blue) and Ptc (red). (**B**,**C**) Graphs representing the number of Ptc-expressing cells (B) and the intensity of Ptc expression (C) in the presence or absence of Dally. (**D**,**E**) Graphical representation of En (D) and Ptc (E) expression inside or outside of the mutant clone shown in A-A″. (**F-F‴**) Overexpressing clones of GFP::Dally (green) labelled for En (blue) and Ptc (red). (A,A′*a*, A′*b*,D) En expression is reduced in both width and intensity in *dally*^{32} mutant clones. (A,A″*c*,A″*d*,B,C,E) The width of the Ptc expression is not reduced (see also a double arrow in E), whereas the intensity of the Ptc is reduced with smaller vesicles in *dally*^{32} mutant clones. (F-F‴) An increase in Ptc vesicles within GFP::Dally overexpressing clones is observed (see magnifications in F″ and F‴ of the white square in F′). Wing imaginal discs are oriented with anterior to the left and dorsal down; green dotted lines delineate the mutant clones and white dotted lines mark A-P boundaries. n, number of clones or discs analysed. Error bars represent s.e.m.

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 *Notum*. Moreover, S2R+ cells express a lower level of *ptc* than Cl8, allowing us to investigate the role of Ptc in Notum and Dally function.

In transfected Cl8 cells, Dally^{Flag} mostly localised to the PM and to a few vesicles (average of 1.2 per cell; Fig. 8G, *1*). These did not colocalise with the Golgi markers GMAP (Friggi-Grelin et al.,

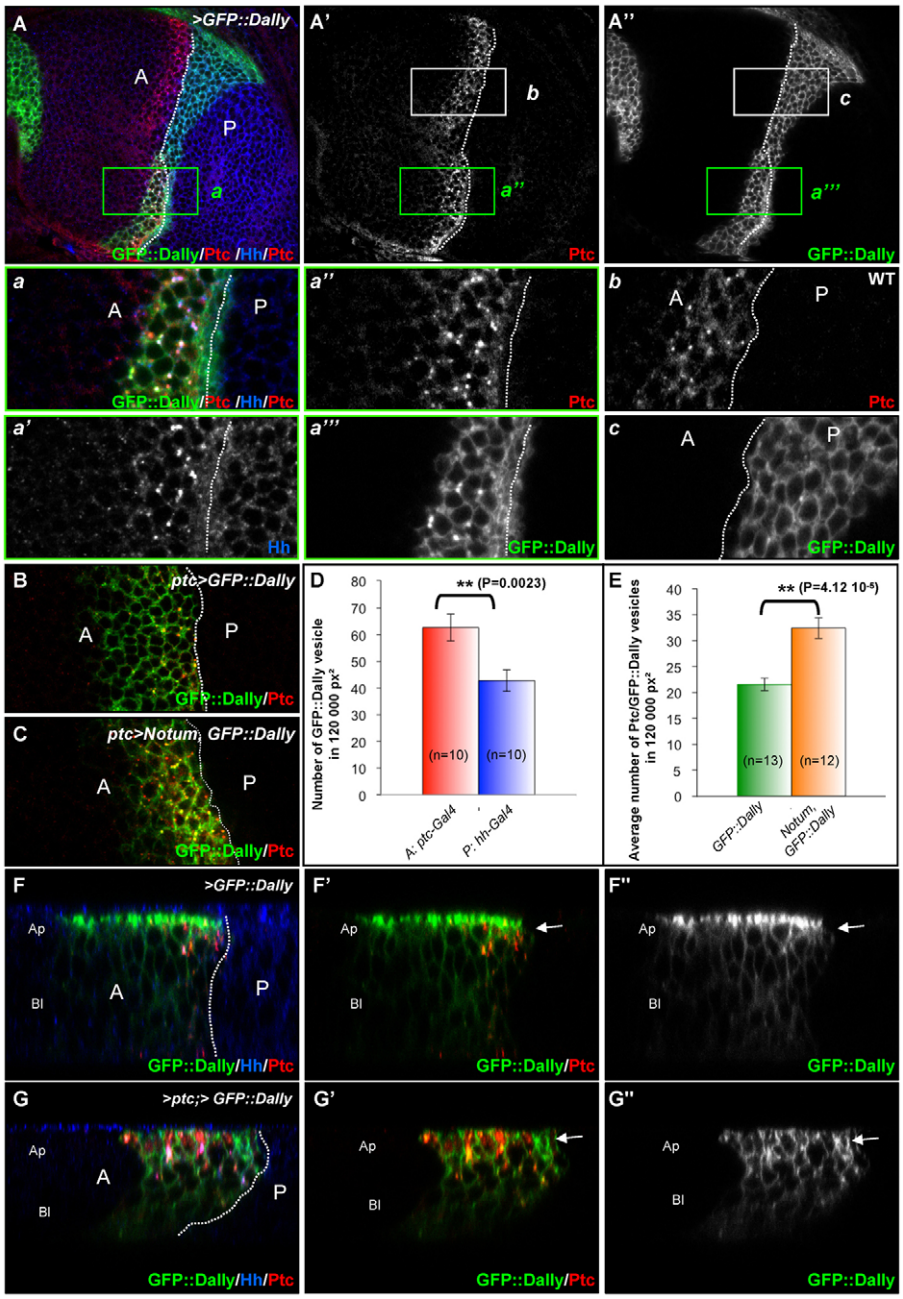

Fig. 7. Dally is internalised with the reception complex, an event that is promoted by Notum. (**A-A″**,***a-a‴***,***b***,***c***,**F-F″**) Clones overexpressing GFP::Dally. (**B**) *ptc-Gal4>GFP::Dally*. (**C**) *ptc-Gal4>Notum; GFP::Dally*. (**D**) Graph of GFP::Dally vesicle numbers in the A compartment (*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.

2006) 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 Dally^{Flag}, we observed a fourfold increase in Dally-containing puncta, and many of these also contained Notum (Fig. 8B-B″,G, *4* compared with *1*). In S2R+ cells, Dally was also found both at the PM and in a few vesicles (Fig. 8C,C′,G, *2*). When Notum and Dally were co-expressed in these cells, we found only weak changes in Dally subcellular localisation (Fig. 8D,D′,G, *5*). Surprisingly, in these cells Notum was localised to both internal vesicles and also at the PM in 50% of the cells observed (Fig. 8D″). One difference between the two cells types is the level of Ptc expression, which is considerably lower in S2R+ cells and which may explain discrepancies between the effects of Notum on Dally subcellular localisation between these two cell types. To test this, we co-transfected S2R+ cells with both Dally and Ptc, which did not largely affect the number of Dally vesicles observed (Fig. 8E,G, *3*). However, introducing Notum augmented vesicle number to a level similar to that observed in Cl8 cells doubly transfected with Dally and Notum (Fig. 8F,G, *6*). This strongly suggests that Notum regulates the intracellular levels of Dally, through regulation of GPI cleavage and Ptc-dependent internalisation of an Hh reception complex that includes Dally.

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

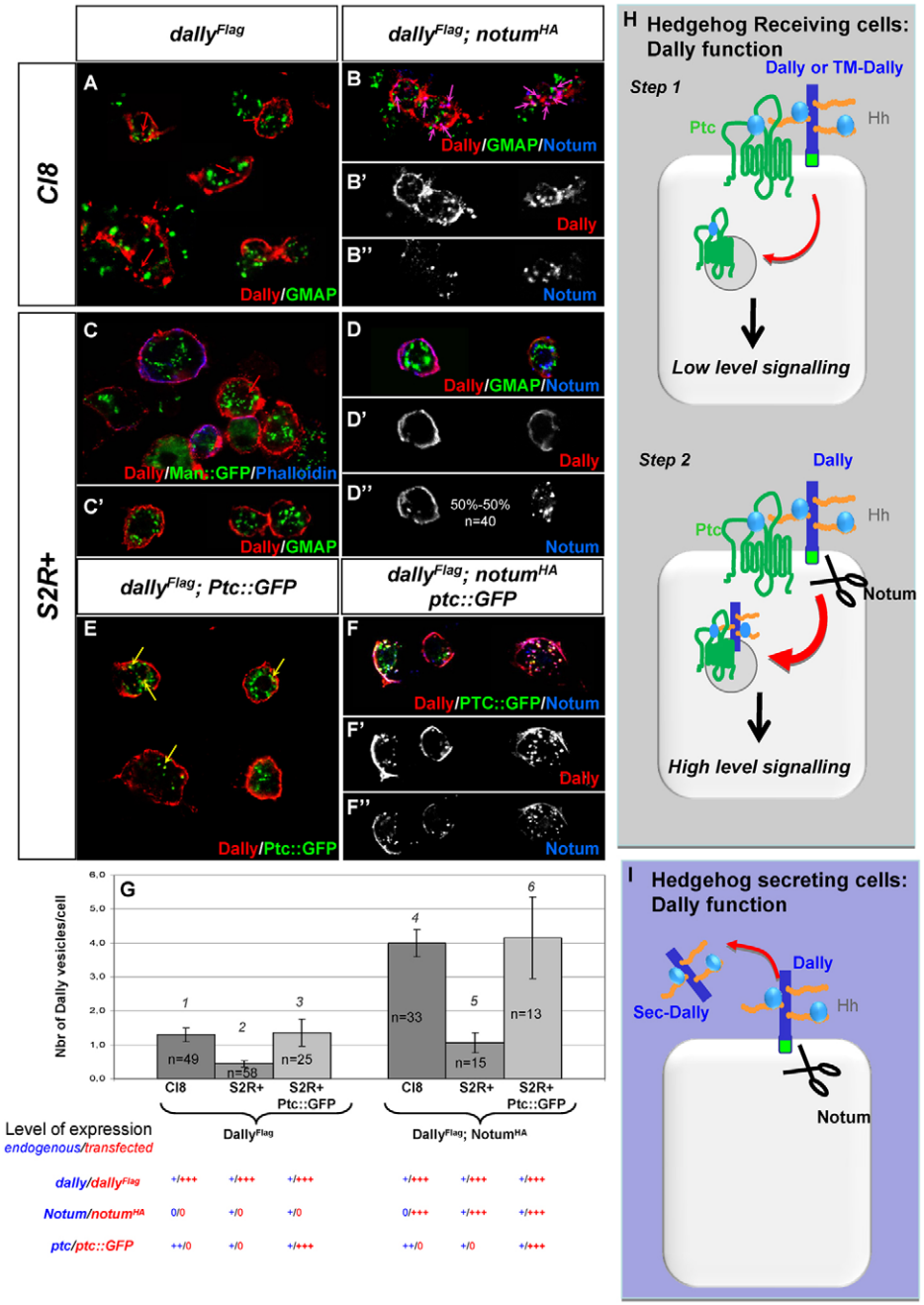

Fig. 8. Notum and Ptc promote Dally internalisation in Cl8 and S2R+ cells. (**A-B″**) Cl8 cells transfected with Dally^{Flag} (A) or Dally^{Flag} + Notum^{HA} (B-B″). (**C-F″**) S2R+ cells transfected with Dally^{Flag} + Manosidase::GFP (a Golgi marker) (C); Dally^{Flag} (C′); Dally^{Flag} + Notum^{HA} (D-D″); Dally^{Flag} + Ptc::GFP (E); or Dally^{Flag} + Notum^{HA} + Ptc::GFP (F-F″). Cells are labelled with anti-Dally (red), anti-GMAP (a Golgi marker; green in A, B, C′ and D), anti-HA (blue in B, D and F), phalloidin (blue in C). (**G**) Graph representing Dally vesicle numbers in the different transfections. The table below represents the relative levels of expression of either endogenous (blue) or transfected (red) genes of interest. Dally is mainly located at the plasma membrane in Cl8 (A) and S2R+ (C-E,G) cells in the absence of Notum and/or at low levels of Ptc. The presence of a high level of Ptc and Notum increases Dally internalisation in vesicles in both cell types (B-B″,F,G). (**H**) A scheme showing a two-step process of Dally-mediated high level Hh pathway signalling. Step 1: Dally mediates Hh sequestration to the receiving cells, perhaps increasing interaction of this ligand with its receptor complex, including Ptc. This role of Dally is independent of the GPI anchor and can be performed by exogenously introduced TM::Dally, which only leads to low level activation of signalling. Step 2: Modification of Dally GPI by Notum, perhaps representing GPI hydrolysis, leads to increased internalisation of Dally with Hh and the reception complex, and contributes to high level Hh signalling. (**I**) The differential functions of Dally and Notum in Hh-secreting cells. The absence of Ptc in these cells means that upon GPI cleavage by Notum, Dally is released from the cells and mediates Hh long-range gradient formation.

(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 shedding 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. 8H,I). 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 reception 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

Supplementary material available online at
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