Development 133, 407-418 doi:10.1242/dev.02212

Cholesterol modification is necessary for controlled planar long-range activity of Hedgehog in *Drosophila* epithelia

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The Hedgehog morphogen is a major developmental regulator that acts at short and long range to direct cell fate decisions in invertebrate and vertebrate tissues. Hedgehog is the only known metazoan protein to possess a covalently linked cholesterol moiety. Although the role of the cholesterol group of Hedgehog remains unclear, it has been suggested to be dispensable for the its long-range activity in *Drosophila*. Here, we provide data in three different epithelia – ventral and dorsal embryonic ectoderm, and larval imaginal disc tissue – showing that cholesterol modification is in fact necessary for the controlled long-range activity of *Drosophila* Hedgehog. We provide an explanation for the discrepancy between our results and previous reports by showing that unmodified Hh can act at long range, albeit in an uncontrolled manner, only when expressed in squamous cells. Our data show that cholesterol modification controls long-range Hh activity at multiple levels. First, cholesterol increases the affinity of Hh for the plasma membrane, and consequently enhances its apparent intrinsic activity, both in vitro and in vivo. In addition, multimerisation of active Hh requires the presence of cholesterol. These multimers are correlated with the assembly of Hh into apically located, large punctate structures present in active Hh gradients in vivo. By comparing the activity of cholesterol-modified Hh in columnar epithelial cells and peripodial squamous cells, we show that epithelial cells provide the machinery necessary for the controlled planar movement of Hh, thereby preventing the unrestricted spreading of the protein within the three-dimensional space of the epithelium. We conclude that, as in vertebrates, cholesterol modification is essential for controlled long-range Hh signalling in *Drosophila*.

KEY WORDS: Hedgehog morphogen, Cholesterol, Activity, Epithelia, *Drosophila*

INTRODUCTION

Hedgehog (Hh) family genes encode secreted peptides that play crucial morphogenetic roles during vertebrate and invertebrate embryogenesis. Hh is a versatile protein that acts both at short range to direct the development of juxtaposed cells as well as at a distance to influence cells located far from its source of production. As a morphogen, Hh determines cell fate behaviour in a concentration-dependent manner. Its importance is underscored by the fact that disorder in its movement severely disrupts the global organisation of numerous tissues during development (reviewed by Ingham and McMahon, 2001).

Extensive research has investigated the mechanisms by which Hh travels through tissues to affect the development of nearby and distant cells. It is clear that the movement of Hh within tissues is tightly controlled and cannot be simply explained by free diffusion. To date, several types of molecules have been identified as playing a role in this process.

Dispatched (Disp), is a Sterol Sensing Domain (SSD)-containing protein that is required for the apical targeting of Hh in epithelial cells (Gallet et al., 2003). This localisation appears to be important because, in the absence of Disp function, cholesterol-modified Hh is not secreted and remains localised to the basolateral membrane. When this happens, Hh cannot reach distant cells, and the only cells that remain even slightly responsive to the Hh signal in *Drosophila* and in some vertebrate tissues are those juxtaposing the Hh source (Amanai and Jiang, 2001; Burke et al., 1999; Caspary et al., 2002; Gallet et al., 2003; Kawakami et al., 2002; Ma et al., 2002; Nakano et al., 2004; Tian et al., 2005). Although the exact function of Disp remains unknown, it has been suggested to be a proton gradient-driven transporter of the RND family, as mutations in conserved residues impair its activity (Ma et al., 2002).

Other molecules involved in Hh movement are the heparan sulfate proteoglycans (HSPG). In *Drosophila*, this family includes the glypicans Dally and Dally-like (Dly), and recent data have suggested that Dally and Dly are substrates for the heparan sulfate polymerase Tout velu (Ttv), which is required for the synthesis of heparan sulfate glycosaminoglycan (HS GAG) chains (Baeg et al., 2001; Han et al., 2004b; Lin and Perrimon, 1999; Tsuda et al., 1999). Absence of these HSPGs is sufficient to block distribution and transmission of the Hh signal to adjacent wild-type cells, strongly suggesting that HSPG are necessary for Hh movement (Bellaiche et al., 1998; Bornemann et al., 2004; Gallet et al., 2003; Han et al., 2004a; Takei et al., 2004).

A particularly intriguing issue that is likely to be key to understanding the developmental function of Hh is the role that its lipophilic anchors play in controlling its long-range activity through tissues. Hh is synthesised as a precursor that, following auto-cleavage yields an N-terminal signalling secreted peptide that is covalently linked to a cholesterol molecule at its C terminus. This signalling Hh peptide (termed Hh-Np, with 'p' standing for 'processed') is further modified by palmitoylation on the first cysteine, both on *Drosophila* Hh and on its vertebrate orthologue sonic hedgehog (Shh). Hh is the only known metazoan protein with a covalently linked cholesterol moiety. Earlier studies have shown that the cholesterol group plays a role in the membrane retention of the protein (reviewed by Mann and Beachy, 2004).

The role of cholesterol in the control of Hh long-range activity appears to be different in *Drosophila* and in vertebrates. In flies, a Hh peptide that is devoid of cholesterol (named Hh-N) has been described as being able to fulfill all Hh-Np functions. Moreover, it

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Accepted 14 November 2005

can induce the expansion of Hh-responsive cells and bypass the requirement for Disp function in its secretion and long-range activity through the *Drosophila* wing imaginal disc (Burke et al., 1999). By contrast, in vertebrates, the non-cholesterol-modified Hh form (Sonic Hh-N or Shh-N) is unable to act at a distance from its source and is thus unable to rescue Shh-dependent long-range activity in the limb bud (Lewis et al., 2001).

It is unclear how Hh-Np, which is membrane-tethered through its cholesterol adduct, can reach distant cells. One possible answer may involve large punctate structures (LPSs), which are formed by Hh-Np, but not Hh-N, in *Drosophila*. Because their formation and movement require Disp and Ttv activity respectively, we have proposed that LPSs provide a vehicle for Hh long-range activity (Gallet et al., 2003; Gallet and Thérond, 2005). Interestingly, in cultured cells, Shh-Np multimerises, but Shh-N does not (Chen et al., 2004; Zeng et al., 2001), suggesting that Hh-LPSs might also depend on Hh-Np multimerisation in flies. Multimerisation of Shh-Np has been proposed to be required for its solubility and long range activity. Alternative mechanisms are currently being explored and several types of vehicles for Hh/Shh have been proposed. For example, a few percent of total *Drosophila* Hh have been identified in lipoprotein particles (Panakova et al., 2005) that might resemble vertebrate low-density lipoprotein or LDL. It has also been shown that Shh, on the surface of mouse ventral node, is packed in membrane vesicles called nodal-vesicular-parcels (Tanaka et al., 2005).

How can we reconcile the contradictory data in *Drosophila* and vertebrates concerning the requirement of cholesterol for the long-range activity of Hedgehog? Here, in three different assays, we clearly demonstrate the existence of Hh-Np long-range activity in the ventral and dorsal embryonic ectoderm and in the imaginal discs of *Drosophila*. These assays provide evidences that, in the absence of cholesterol modification, Hh is devoid of controlled long-range activity in embryonic or in imaginal disc cells, and explain previous published data from Burke and colleagues (Burke et al., 1999). Furthermore, we show that whereas Hh-Np dimerises and forms multimers, Hh behaves as a monomer in the absence of its cholesterol moiety. Fractionated Hh-Np multimers aggregate at the plasma membrane similar to Hh-LPSs and have full Hh activity. We also demonstrate that the lack of cholesterol affects both the intrinsic activity of Hh and the routing of its secretion. By expressing cholesterol-modified Hh in columnar epithelial cells or in peripodial squamous cells, we show that epithelial cells provide the machinery necessary for the controlled planar movement of Hh. Based on these data, we propose that the cholesterol adduct is necessary to prevent the unrestricted spreading of Hh within the three-dimensional space of the epithelium.

MATERIALS AND METHODS

Drosophila stocks and genetic experiments

hh^{AC}, *FRT82B* $disp^{377}$and *FRTG13* ttv^{00681} are null alleles (Burke et al., 1999; Lee et al., 1992; The et al., 1999). Two lines of *UAS-hh-Np* [*UAS-hhM1* and *UAS-hhM4* (Ingham and Fietz, 1995)] and two lines of *UAS-hh-N* (Gallet et al., 2003; Thé et al., 1999) were used. *UAS-hh-N-CD2*, which corresponds to a fusion of the transmembrane domain of the rat CD2 protein with the Hh-N peptide, has been described previously (Strigini and Cohen, 1997). *UAS-*$disp^{HA}$ (Burke et al., 1999), *UAS-C85S-hh-N* (Gallet et al., 2003) and *UAS-*shi^{DN} (Moline et al., 1999) was as previously described. Other stocks were obtained from the Bloomington stock center. *disp*-null and *ttv*-null embryos were obtained by generating germline clones using a standard protocol (Chou and Perrimon, 1996) and performed as described previously (Gallet et al., 2003). Recombinant chromosomes hh^{AC} *UAS-hh-Np,* hh^{AC} *UAS-hh-N-CD2* and hh^{AC} *UAS-C85S-hh-N* were described previously (Gallet et al., 2003). All *UAS-Gal4* experiments (Brand and Perrimon, 1993) were performed at 25°C (except for Fig. S1C,D, which were carried out at 29°C). Overexpression of clones in the wing imaginal discs was produced using the 'flip-out' technique (Basler and Struhl, 1994) on heat shocked early third instar larvae hatched from *dpp-lacZ; act<CD2<Gal4, UAS-GFPnls* females crossed to *yw hs-flp122; UAS-hh-N* or *yw hs-flp122, UAS-hh-Np* or *yw hs-flp122; UAS-hh-N-CD2* males. About 900 clones for each flip-out experiment were analysed per genotype.

For hh^{AC} clones in the P compartment expressing either Hh-Np or Hh-N, the following strains were used: females *yw hs-flp; UAS-hh-MI; FRT82B* hh^{AC}*/TM6B* or *yw hs-flp; UAS-hh-N; FRT82B* hh^{AC}*/TM6B* were crossed to *w, enGal4, FRT82 tubGal80* males. F1 progeny were heat shocked for 80 minutes at 38°C 24 hours after hatching and then third instar larvae were fixed and stained. *disp* mutant wing imaginal discs were prepared from homozygous $disp^{377}$/$disp^{377}$ third instar larvae. In Fig. 2L, 15 independent A4 and/or A5 dorsal segments for each genotype (except for the wild type, where only eight were counted) were captured with a numeric camera and the length covered by type 2 and 3 cuticles was measured using PhotoShop imported images.

Cuticle preparation, in situ hybridisation and immunostaining

Cuticle preparation, immunostaining and in situ mRNA hybridisation was performed as described previously (Gallet et al., 2003). Antibodies were used at the following dilutions: mouse 4D9 monoclonal anti-En (gift from M. Bourouis) and mouse 4D4 monoclonal anti-Wg at 1/20 (Developmental Studies Hybridoma Bank University of Iowa); rabbit 'Calvados' polyclonal anti-Hh at 1/200 (Gallet et al., 2003); rat anti-BiP at 1/400 (Babraham Bioscience Technologies); monoclonal mouse anti-βGal at 1/1000 (Promega); monoclonal rabbit anti-βGal at 1/500 (Cappel); alkaline phosphatase-coupled anti-Fluo at 1/500; or anti-Dig at 1/1000 (Roche). Fluorescent secondary antibodies were used at 1/200 for Cy3-conjugated donkey anti-rat, Cy5-conjugated goat anti-mouse and Texas-Red-conjugated goat anti-mouse (Jackson laboratory), and 1/500 for Alexa Fluor 488-conjugated anti-rabbit (Molecular Probes). Secondary biotin-conjugated (at 1/1000, Jackson laboratory) and streptavidin Alexa Fluor 488-conjugated (at 1/200, Molecular Probes) were used to amplify Hh immunostaining in embryos. Vector kits were used for peroxidase staining. The Roche NBT/BCIP reagent was used for blue labelling of mRNA in in situ hybridisations. Red fluorescent in situ hybridisation was obtained using the Vector Red Alkaline Phosphatase Kit I (SK5100, Vector Laboratories). Fluorescent images were obtained from a Leica Sp DMR TCS_NT confocal microscope and processed using Adobe PhotoShop 7.0.

Cell culture

The antibody against Fu is described elsewhere (Ruel et al., 2003). *Drosophila* Schneider line-2 and wing imaginal disc cl-8 cells were maintained as described previously (van Leeuwen et al., 1994). cDNAs encoding wild-type Hh and Hh-N were cloned by PCR into the *Drosophila* inducible expression vector pMT/V5-His (Invitrogen) and transfected into cl8 cells by Cellfectin (Invitrogen). Selection of stably transformed cl-8 cell lines was performed using hygromycin selection (Cumberledge and Krasnow, 1993). Cl8 cells expressing different Hh constructs were grown on chamber slides (with 0.1 M cadmium for induction of Hh expression). After induction, the cells were fixed, permeabilised (or not) with Triton X100, immunostained for Hh and BiP (ER marker), and mounted onto slides for confocal analysis.

Gel filtration chromatography and Hh assays

Serum (1%) or serum-free conditioned media from Hh-Np or Hh-N cultures were collected, ultracentrifuged (100,000 ***g*** for 30 minutes), and filtered through a 0.22 μm filter. The supernatants were loaded on Sephacryl S-200HR (Pharmacia) or Superose 6 (Pharmacia) gel filtration columns. These columns cover a wide molecular weight range (5-250 kDa for Sephacryl S-200HR; 5-5000 kDa for Superose 6) and were equilibrated with serum-free media. All the eluted fractions were precipitated with TCA and immunoblotted with antibodies against Hh. In Fig. 3D,E, naive cl8 cells transfected with the *8X gli-luciferase* reporter gene in chamber slides were incubated with different pooled fractions of Hh for 2 hours and immunostained for Hh or assayed for luciferase activity. In Fig. 3G, wild-

type embryonic extracts (0-12 hours) were collected, dechorionated, lysed in PBS/0,05% NP-40 with a Dounce homogeniser and treated similarly to the Cl8 cells, except that the columns were equilibrated with PBS/0,05% NP-40 and the fractions were immunoprecipitated with a Hh antibody.

Immunoprecipitation

S2 cells were co-transfected with expression constructs encoding actin-Gal4 and different *UAS* plasmids: *UAS-hh-Np, UAS-hh-N* and *UAS-hh-HA-Np* (Hh-Np with HA tag, a gift from K. Basler). Cells transfected with Cellfectin (Invitrogen) were incubated in serum-free medium. Two days later, the Hh conditioned medium was harvested and transfected S2 cells lysed in lysis buffer (PBS/0,05% NP-40). For HA immunoprecipitation, 10 μl of protein G-Sepharose were pre-bound to mouse monoclonal antibodies (anti-HA) and added to the clarified cell lysates or Hh conditioned medium at 4°C for 2 hours. Immunocomplexes were washed four times with lysis buffer and resolved by SDS-PAGE. Enhanced chemoluminescence reagents (Amersham) were used for antibody detection following blotting to nitrocellulose membranes.

RESULTS

Identification of a long range Hh activity in the ventral ectoderm

The existence of a long-range activity for Hh has never been demonstrated in *Drosophila* embryos. Investigating this issue has proved difficult because of the lack of clear molecular targets that are distant from the Hh source. In the ectoderm, Hh is expressed under the control of Engrailed (En) and forms apical LPS necessary for maintenance of *wingless* (*wg*) in cells anterior to En cells (Gallet et al., 2003). Apical Hh-LPSs form a symmetric gradient 3 or 4 cells wide on both sides of their source (Fig. 1A′), when compared with the asymmetric gradient formed by Wg (Fig. 1A′′) (Dubois et al., 2001). In the course of our study, we observed that Hh-LPSs are present far from their source (Fig. 1A,A′) (see also Gallet and Thérond, 2005), suggesting that Hh might regulate the expression of genes at distance from its source. One candidate is the *serrate* (*ser*) gene, which is expressed at stage 12 of embryogenesis in three rows of cells (Thomas et al., 1991) at a similar distance from the two Hh sources that are present in adjacent segments.

We have previously showed that Hh-LPS distribution is strongly restricted at stage 11 in comparison with earlier stages (Gallet and Thérond, 2005) and that *ser* expression and Hh-LPS staining are mutually exclusive: *ser* expression initiates where Hh-LPSs spreading begins to be restricted (Fig. 1A,A′). Furthermore, modulating the expression level of Hh-Np alters the width of the domain of *ser* expression. For example, moderately increasing Hh-Np expression in En cells restricted *ser* expression to one or two rows of cells (Fig. S1B), and high levels of Hh-Np expression completely abolished *ser* expression independently of Wg activity (Fig. S1C,D). Because Hh signalling regulates the maturation of a transcriptional activator, Cubitus interruptus (Ci) and because overexpression of Ci repressed *ser* (Alexandre et al., 1999), we propose that Hh affects *ser* expression indirectly through the activity of a transcriptional repressor.

To confirm that the anterior and posterior boundaries of *ser* expression are indeed defined by the range of Hh-LPS activity, we analysed the activity of the non-cholesterol-modified Hh-N peptide and the membrane bound Hh-N-CD2 protein, which are both unable to form LPSs (Gallet et al., 2003). When Hh-N or Hh-N-CD2 constructs were expressed in place of Hh-Np, *ser* expression was no longer repressed and occurred in cells adjacent to the Hh source at the anterior edge (Fig. 1B-C). This difference compared with Hh-

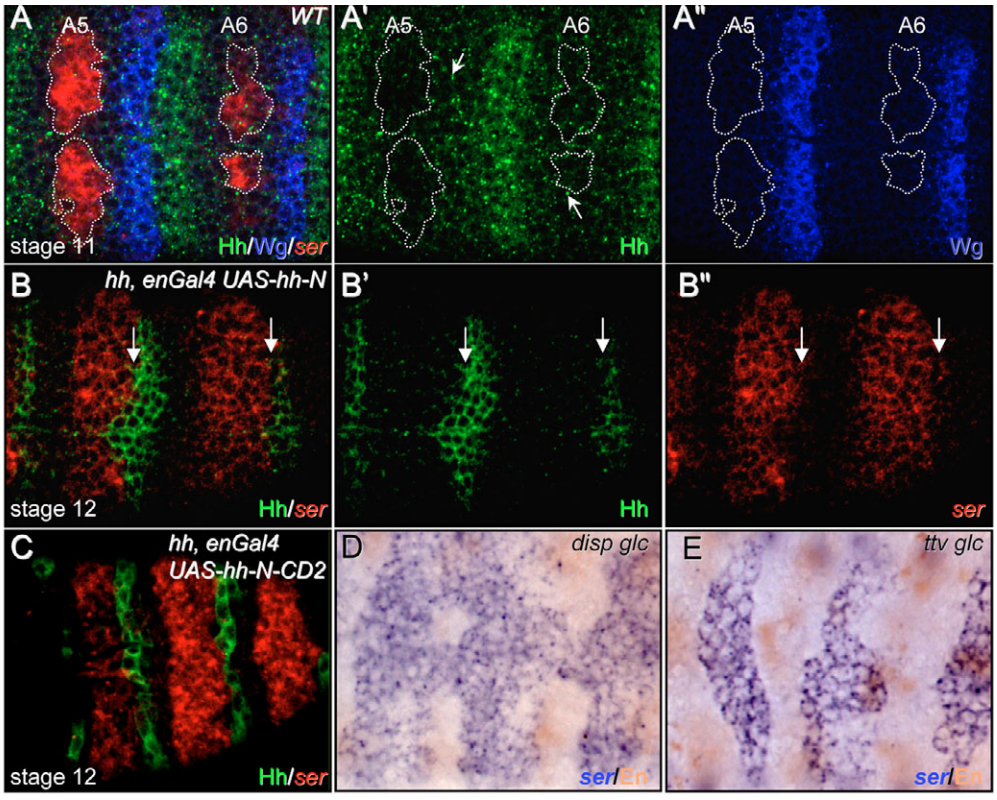

Fig. 1. Hh-Np activity defines the limit of *serrate* expression in the ventral ectoderm. In all panels anterior is towards the left. (A-A′′) Wild-type embryos. (B-B′′) hh^{AC}, *engal4 UAS-hh-N* embryos. (C) hh^{AC}, *engal4 UAS-hh-N-CD2* embryos. (D) *disp* and (E) *ttv* germline clone embryos. In A-C, *ser* mRNA is in red, Hh protein is in green and Wg protein is in blue; in D,E, *ser* mRNA is in blue and En protein is in brown. (**A**-A′′) In wild-type stage 11 embryos, the Hh-Np gradient is symmetric, when compared with the asymmetric Wg gradient. Hh-Np LPSs are detected at a distance of three or four cells from their source (arrows in A′), and very few Hh-Np LPSs are detected within the *ser* expression domain (outline in A-A′′). No LPS-like structures are detected in Hh-N-expressing embryos (**B**,B′), and *ser* is expressed broadly and juxtaposed with the Hh-N source (arrows in B-B′′ indicate the boundary between Hh-N- and *ser*-expressing cells). (**C**) When Hh-N-CD2 replaces endogenous Hh, *ser* is no longer repressed. (**D**,**E**) In the absence of any *disp* or *ttv* function, *ser* expression is no longer repressed. Derepression of *ser* expression is observed in *ttv* mutant to a lesser extent than in *hh* or *disp* mutants. This could be due in part to the remaining *rho* expression, which is independent of Ttv activity and of Hh-LPS movement (Gallet et al., 2003).

Np activity is not due to a difference in the level of expression of the transgenes, as they expressed Hh proteins at comparable levels in these embryos (Fig. S2 in the supplementary material). Similarly, in *disp*-mutant embryos, in which no Hh-LPSs are formed, *ser* was no longer repressed (Fig. 1D). Moreover, in *ttv*-mutant embryos, in which Hh-LPS movement is impaired, *ser* was also derepressed (Fig. 1E).

We should point out that *ser* derepression is incomplete in Hh-N or Hh-N-CD2-expressing embryos. For example, enGal4-driven expression of Hh-N causes the repression of *ser* expression in the first three or four cells posterior to the Hh-N expression domain. In fact, both Hh-CD2 and Hh-N induced *rho* in cells posterior to Hh (see Fig. S3 in the supplementary material). Because *ser* and *rho* are mutually exclusive, the expansion of *ser* derepression is limited to the non-*rho* expressing cells in these embryos. This is reminiscent of our earlier work showing that cholesterol modification is not needed for signalling towards the posterior (Gallet et al., 2003). In any case, the domain of *ser* repression posterior to the Hh-N-expressing domain is no greater than that observed in Hh-Np expressing animals (Figs S1, S3).

Taken together, our results show that *ser* is a bona fide long-range target repressed by Hh that can be used to follow the limits of the range of Hh action within the ventral ectoderm. It also demonstrates that the formation and long-range activity of Hh-Np LPSs are necessary for setting the limits of expression of this newly identified long-range target (Fig. S1E).

Hh-N is likely palmitoylated at its N terminus

The difference in the ranges of activity of Hh-N and Hh-Np could be explained by a difference in the potency of the two molecules. The potency of the Hh signal is dependent on the addition of palmitate at the N terminus: this second lipid modification is crucial for the full activity of the signal, both in vitro and in vivo. Because the efficiency of palmitate addition on Shh has been shown in High Five cultured cells to depend on prior processing and cholesterol addition (Pepinsky et al., 1998), it is possible that our Hh-N construct not only removes cholesterol but also reduces the efficiency of palmitate addition. Therefore, we compared the activity of the non-cholesterol-modified Hh form (Hh-N) with that of a double-mutant form of Hh that cannot be palmitoylated nor cholesterol modified (C85S-Hh-N). Interestingly, Hh-N was inactive when not palmitoylated, strongly suggesting that a significant fraction of the truncated Hh-N construct used in our experiments is palmitoylated in vivo (Fig. S3) (Chamoun et al., 2001). Similar results have been obtained with Shh lacking both lipids [C24S-ShhN (Feng et al., 2004)]. Therefore, if Hh-N has a decreased potency this can most likely be attributed to the lack of cholesterol (see below).

Cholesterol-modification of Hh is required for its long-range activity in the embryonic dorsal epidermis

The embryonic dorsal ectoderm is another model for studying the long-range activity of Hh (Bokor and DiNardo, 1996; Heemskerk and DiNardo, 1994). Four types of cuticle develop dorsally (Fig. 2A), with type 1 cells being the En/Hh-expressing cells and type 4 being the only cell fate that develops in a *hh* mutant embryo (Fig. 2B). Based on experiments involving the modulation of Hh expression levels, it has been proposed that Hh specifies cell fates 1 to 3 in a concentration-dependent manner, although the existence of a potential second signal that acts to relay Hh activity has not been definitively ruled out (Heemskerk and DiNardo, 1994).

To determine which cell types are directly dependent on Hh, we expressed the Hh-N-CD2 transgene in the *en* expressing cells and tested its ability to rescue a loss of *hh* function. Although Hh-Np rescued all dorsal cuticle (Fig. 2C), Hh-N-CD2 only rescued type 1 and adjacent type 2 cuticles, but not the distant type 3 cuticle (Fig. 2D). In addition, the type 2 cuticle domain was more restricted than it was in wild-type embryos. These results demonstrate that the type 1 to 3 cell fates depend directly on the Hh gradient and not on a relayed signal. Type 1 cuticle is probably determined by the highest Hh level, whereas the lowest Hh level specifies the distal type 3 cuticle fate.

To address the role of cholesterol modification, Hh-N activity was measured in a similar way. We found that it induced only type 2 cuticle, and no type 3 cuticle, when expressed in *en* cells (Fig. 2E). This suggests that Hh-N long range activity is restricted in comparison to that of Hh-Np. When the total distance encompassing both type 2 and type 3 is calculated in these embryos, the range of activity of Hh-N is about half that of Hh-Np (Fig. 2J,K).

In *disp*-mutant embryos, only type 4 cells were observed, as in *hh*-null embryos (Fig. 2G). Expression of exogenous Hh-Np in *en* cells of *disp*-null embryos rescues some type 1 cuticle (arrows in Fig. 2H), most probably owing to an autocrine activity of Hh-Np. The absence of type 2 and 3 cuticles confirms the requirement of Hh movement for patterning the dorsal epidermis. When Hh-N was expressed in *en* cells of *disp*-null mutant embryos, some type 2, but no type 3, cuticle was formed (Fig. 2I). This conforms to previous results showing that Disp is not required for regulating the secretion of the non-cholesterol-modified Hh-N (Gallet et al., 2003). Unfortunately, we could not assess the function of Ttv in regulating dorsal cell fates, because the dorsal cuticle is absent in *ttv* glc embryos (data not shown) owing to the requirement of Ttv for other signalling pathways (Lin, 2004).

Could the reduced long-range activity of Hh-N be a consequence of a reduced potency of the molecule? If this was the case, one would expect an overall range of Hh-N activity similar to that in wild-type embryos, but with an enlarged type 3 domain width at the expense of type 2 cells that require a highest level of signalling. This was not what we observed (Fig. 2E). In order to analyze the consequence of a reduction of Hh signalling activity, we expressed a dominant negative form of the *Drosophila* dynamin homologue Shibire (Shi^{DN}) in Hh-responsive cells. In this case Ptc internalisation is blocked but Hh spreading is not affected (Gallet and Thérond, 2005; Torroja et al., 2004). In such embryos, we observed that the overall range of Hh activity was similar to that in wild-type embryos, but with an enlarged type 3 domain width at the expense of type 2 cells, as if the overall level of Hh signalling was reduced (Fig. 2F,J-K). This result was the opposite to that obtained with Hh-N, suggesting that the lack of cholesterol adduct mainly affects Hh long-range activity, most likely by reducing its cell membrane association and consequently its planar movement (see below).

Cholesterol promotes full Hh activity through multimerisation

To test if the restricted long-range activity of Hh-N is consequent to the diminution of its membrane association we generated two different *Drosophila* cl8 cell lines in which Hh-Np or Hh-N expression could be induced by the addition of cadmium (Cd). Following induction, Hh-N and Hh-Np were detected in large vesicles within permeabilised cells (Fig. 3A). These vesicles probably correspond to Hh peptides present within the export pathway, as suggested by co-staining with the endoplasmic reticulum marker BiP. In non-permeabilised cells, a condition that

allows the detection only of outer leaflet membrane-localised proteins, Hh-Np expressing cells showed strong punctate labeling on the plasma membrane (arrows in Fig. 3B). Conversely, Hh-N-expressing cells did not display any membrane staining, confirming that the cholesterol modification of Hh increases its affinity for the plasma membrane.

We next analysed the supernatants of serum-free cultured cells by submitting them to gel-filtration chromatography. Similar to what has been observed with Shh, Hh-Np molecules were recovered in the high molecular weight fractions (up to 160 kDa, Fig. 3C, peak A), whereas Hh-N was detected in the low molecular weight fractions close to its predicted molecular weight (Fig. 3C, peak B). The analysis was further performed on a second column that resolves high molecular weight complexes (Fig. 3C, lower panels). We noticed that, in addition to peak A, residual Hh immunoreactivity was detected in higher fractions close to the void volume (Fig. 3C, lower panels), suggesting the possible existence of a higher molecular weight Hh-Np containing complex. When

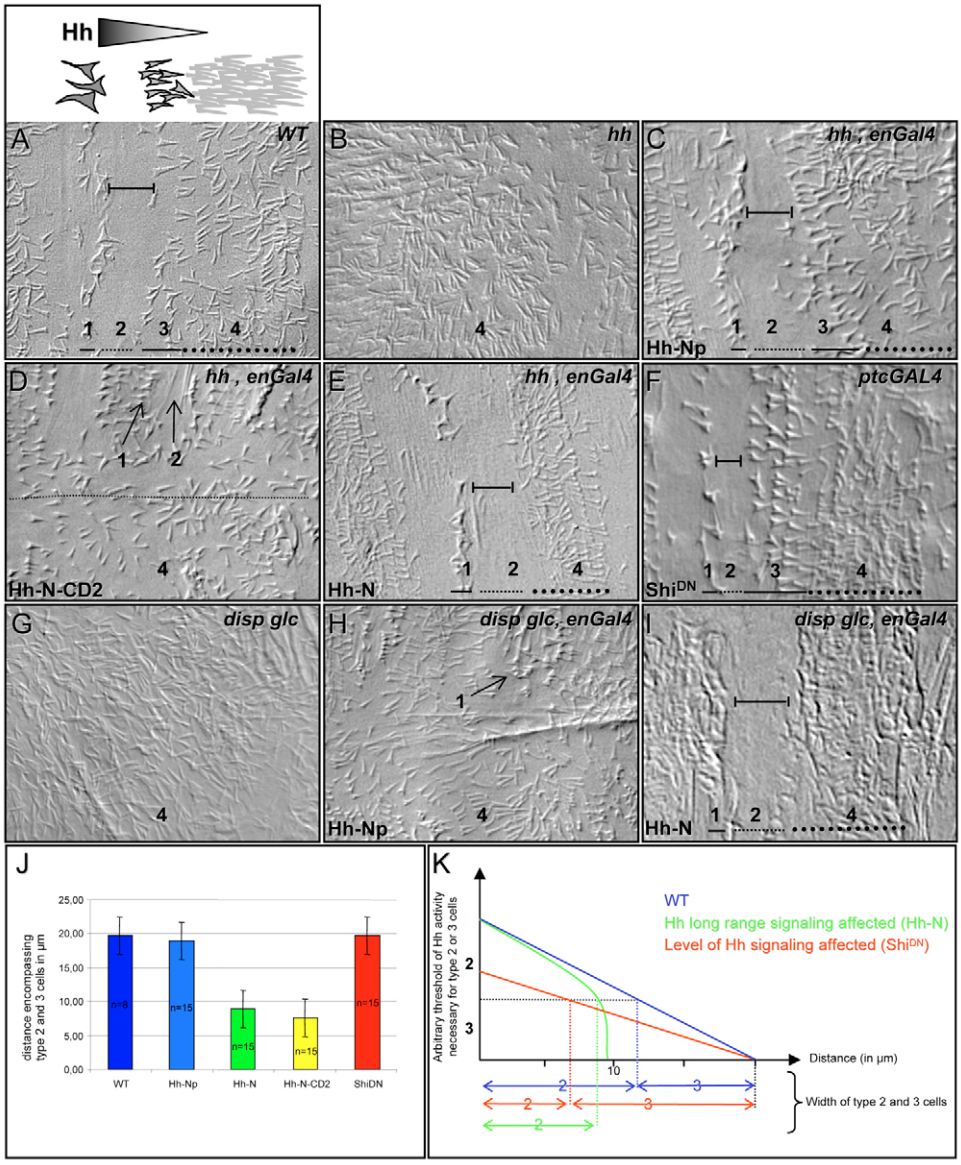

Fig. 2. Cholesterol-modification of Hh is necessary for Hh-dependent graded cell fate specification in the dorsal epidermis. All panels show dorsal cuticle views of first instar larvae. (A) Wild type. (B) *hh*^{AC}. (C) *hh*^{AC}, *engal4 UAS-hh-Np*. (D) *hh*^{AC}, *engal4 UAS-hh-N-CD2*. (E) *hh*^{AC}, *engal4 UAS-hh-N*. (F) *ptcgal4 UAS-Shi*^{DN}. (G) *disp* glc. (H) *disp* glc, *enGal4 UAS-hh-Np* and (I) *disp* glc, *enGal4 UAS-hh-N*. (**A**) In the wild-type dorsal epidermis, Hh is secreted from type 1 cells and forms a morphogen gradient that allows the differentiation of cell types 1 to 3 in a concentration-dependent manner (scheme and cuticle). (**B**) In *hh* loss of function, only type 4 cells are present. (**C**) Expression of Hh-Np in En cells allows the rescue of the *hh* mutant phenotype. (**D**,**E**) Hh-N or Hh-N-CD2 expression induces some type 1 cells and a domain of type 2 cuticle, but in the case of Hh-N-CD2 naked cuticle was never formed at the midline (broken line in D). (**F**) In *ptcgal4 UAS-Shi*^{DN} larvae, the domain of type 2 cells is reduced, whereas the domain of type 3 cells is enlarged. (**G**) In *disp glc* loss of function, only type 4 cells are present. (**H**,**I**) In *disp glc* embryos, Hh-Np only induced some patches of type 1 cells (arrow in H), whereas Hh-N induced type 1 and some type 2 cells. Bars delimit the width of type 2 cuticle. (**J**) Histogram showing the width of the cuticle covered by type 2 and 3 cells in the genotypes shown in A,C-F. (**K**) A theoretical model based on the results shown in J, representing cell fate behaviours as a function of Hh range and activity. Numbers correspond to the type of fate adopted by the dorsal cells related to the distance from Hh-producing cells.

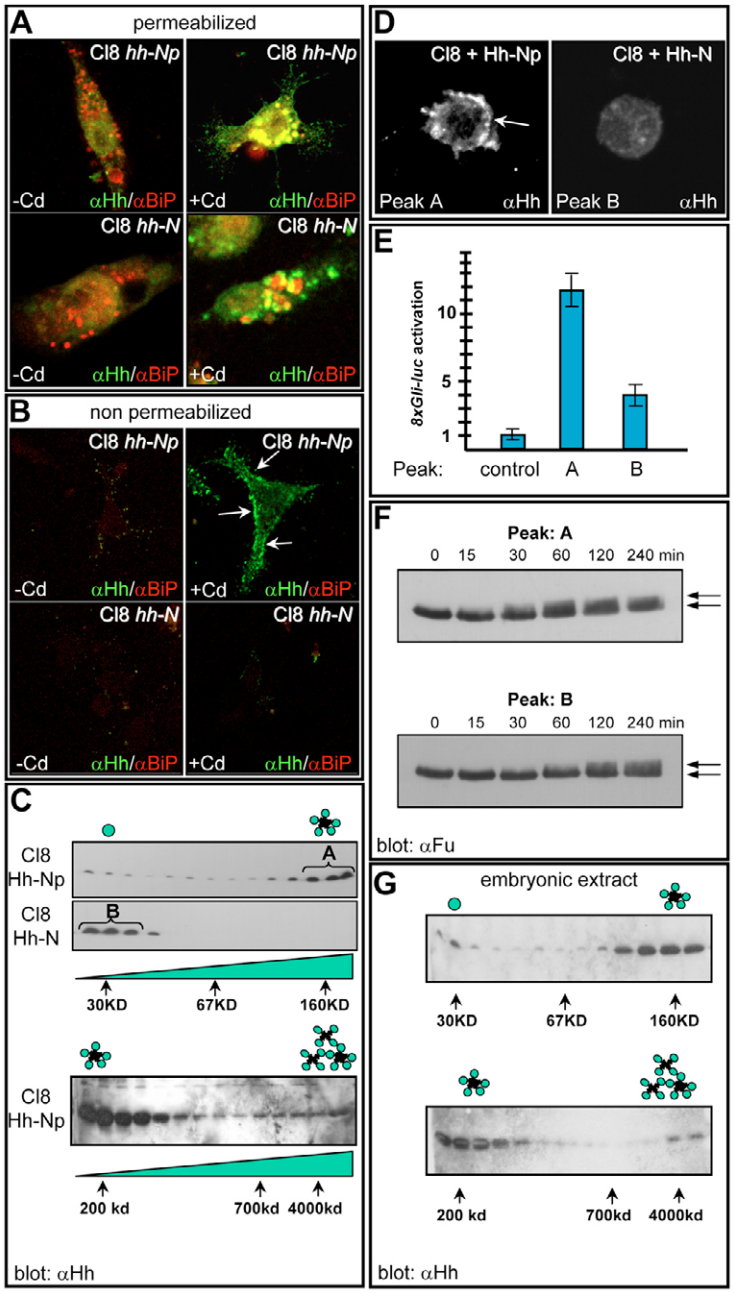

Fig. 3. Cholesterol modification is required for membrane association and full activity of Hh. (**A**,**B**) Cl8 cells were treated with Cd to induce expression of either Hh-Np (top) or Hh-N (bottom). Permeabilised cells (A) or unpermeabilised cells (B) were subjected to immunostaining for Hh (green) and for the ER marker BiP (red). Hh-N and Hh-Np are present in large cytoplasmic vesicles marked with BiP (A). Hh-Np is also present at the plasma membrane (A). In non-permeabilised cells (B), Hh-N was undetectable, while Hh-Np was enriched at the plasma membrane as aggregates (arrows). Even without Cd induction, Hh accretions were faintly detectable because of the leaky promoter. (**C**, top) Media of cultured Cl8 cells expressing either Hh-N or Hh-Np were subjected to gel filtration on a Sephacryl S200 column. Hh-N is recovered only in the low molecular weight fractions corresponding to the Hh monomeric form (peak B). Hh-Np was greatly enriched in the high molecular weight fractions (peak A around 160 kDa). (C, bottom) When subjected to a Superose 6 column, a stable peak of Hh-Np was recovered around 200 kDa. Immunoreactivity was also observed in the highest molecular weight fractions. (**D**) Peak A- or B-containing fractions were applied to naive Cl8 cells that were subsequently fixed and stained for Hh. Hh-N was not detectable, but accretions of Hh-Np were present at the plasma membrane of peak A-treated cells (arrow). (**E**) Both peaks were tested for their ability to induce a Hh reporter gene. At similar concentrations, Hh-Np (peak A) is threefold more potent than Hh-N (peak B). (**F**) When peak A is added to naive cells, it stimulates Fu phosphorylation two to three times faster than peak B (the upper arrow on each blot indicates the phosphorylated Fu isoform). (**G**) Embryonic extracts were subjected to gel filtration on a Superose 6 column: a stable peak of Hh is visible around 200 kDa, but Hh is also present in the very high molecular weight fractions. The last fraction falls outside of the resolution of the column. (C-G) Representative images and blots from three independent experiments.

embryonic extracts were analysed by gel filtration chromatography, Hh-Np migrated at about six times its native molecular weight (Fig. 3G), but was also recovered in the highest molecular weight fractions, similar to what was observed with conditioned medium of Hh-Np or Shh-Np-expressing cells (Fig. 3C) (Feng et al., 2004). When isolated fractions were applied to naive Cl8 cells, we observed strong punctate membrane staining with the Hh-Np containing peak A fractions, and no membrane staining of cells incubated with the Hh-N containing peak B fractions (Fig. 3D).

To compare the activity of the Hh-N monomer with that of Hh-Np multimers, we monitored the relative activity of peak A- and peak B-containing fractions containing similar concentrations of Hh using a luciferase reporter gene (Ruel et al., 2003). As shown in Fig. 3E, Hh-Np containing fractions were threefold more active than were Hh-N containing fractions. Their activities were also analysed by following the kinetics of Fused (Fu) kinase phosphorylation (Therond et al., 1996). Fu phosphorylation was delayed (by 30 minutes) when cells were stimulated with Hh-N fractions versus stimulation with Hh-Np fractions (Fig. 3F).

To demonstrate that the cholesterol moiety is necessary for Hh-Np dimer formation, we transfected *Drosophila* Schneider S2 cells with plasmids expressing Hh-N, Hh-Np or a tagged version of Hh-Np (Hh-HA-Np) (Burke et al., 1999), and carried out immunoprecipitation experiments. We found that Hh-HA-Np and Hh-Np co-precipitated in the culture medium as well as in the cell extract (Fig. 4). Conversely, Hh-N was not present in the immunoprecipitate with Hh-HA-Np when performed either from the culture medium or cell lysates (Fig. 4).

Taken together, our data demonstrate that the cholesterol adduct is required for full Hh activity by providing a cell membrane attachment and the ability to form high molecular weight structures that probably correspond to Hh-Np multimers. Although correlative,

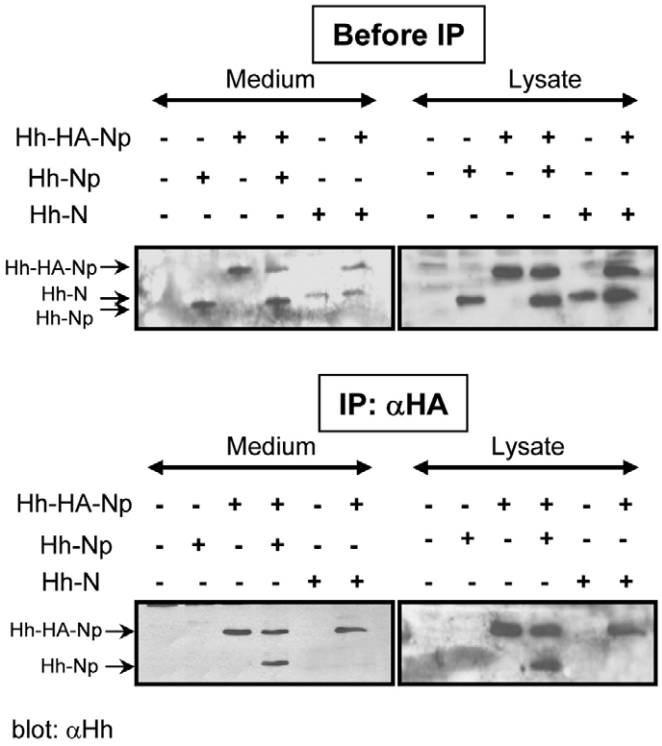

Fig. 4. Cholesterol-modification is required for Hh homodimerisation. Hh-Np- or Hh-N S2-expressing cells were transfected with an HA-tagged form of Hh-Np (Hh-HA-Np). The presence of the Hh proteins in the culture media or in cell lysates was analysed by western blotting (upper panel). The three different Hh forms are easily distinguishable because of their different electromobility properties. Immunoprecipitation using an anti-HA antibody (lower panel) revealed that Hh-HA-Np co-precipitates with Hh-Np both in cell lysates and in culture media, but does not co-precipitate with Hh-N. The HA adduct imposes a low electromobility onto Hh, whereas the cholesterol adduct in Hh-Np is responsible for its higher electromobility when compared with Hh-N.

these data also show that the cholesterol moiety on Hh allows the formation of active soluble structures that can be recognised as accretions at the cell surface similar to the Hh-LPSs observed in the Hh gradient.

Apical Hh-Np LPSs are present in the wing imaginal disc and depend on Disp activity

Because it has been previously shown that cholesterol modification is not necessary for Hh long-range activity in the wing disc (Burke et al., 1999), we decided to switch to this tissue, which permits an analysis of Hh target activation at a much longer range compared with embryonic epithelia. In wild-type imaginal discs, Hh is expressed under the control of the transcription factor En and is secreted from the posterior (P) compartment (Tabata et al., 1992). Hh acts as a morphogen to induce various target genes in the anterior (A) compartment (Basler and Struhl, 1994; Chen and Struhl, 1996; Strigini and Cohen, 1997). The Hh morphogenetic gradient is interpreted by receiving cells through the activity of the transcription factor Ci. In cells receiving high levels of Hh, *en* and high levels of *ptc* are activated, while in cells further from the AP border that are exposed to lower levels of Hh, *decapentaplegic* (*dpp*) is induced (Ogden et al., 2004).

In wing imaginal discs, Hh-Np accretions were apically located (Fig. 5A,C, thin arrows) within the P compartment, as well as in the first rows of A-compartment cells. No apical accretions were observed in *disp* mutant discs (Fig. 5D-F), strongly suggesting that these Hh-Np accretions are sensitive to Disp function, similar to the Hh-LPS in embryos (Gallet et al., 2003). Within the first two or three rows of A-receiving cells, Hh was also present in bigger accretions that contain Ptc and correspond to the internalised Hh-Ptc complex (Martin et al., 2001; Torroja et al., 2004).

Cholesterol modification is required for Hh long-range activity in the wing imaginal disc

To compare the activities of Hh-Np and Hh-N in the wing disc, we generated flip-out clones of cells that are mutant for endogenous *hh* but express either the Hh-Np or Hh-N transgene during larval development (with the use of a Gal80 transgene, see Materials and methods for detailed genotypes). Clones of *hh*-null cells expressing Hh-Np in the P cells that abut the AP boundary activate Ptc expression in six or seven rows of cells (Fig. 5G-I), versus the two or three rows of Ptc-expressing cells in wild-type discs (Fig. 5B,C). Internalisation of Hh-Ptc complexes are readily seen (thick arrow in Fig. 5H,I). Interestingly, in similarly located clones of *hh*-null cells expressing Hh-N, Ptc expression is only induced in three or four rows of cells (Fig. 5J-L). Ptc-Hh complexes are also observable (thick arrows in Fig. 5K,L), although we noticed that a greater number of them are basally located. This location is correlated with a basal enrichment of Hh-N localisation (Fig. 5L) relative to the more apical localisation of Hh-Np (Fig. 5C,I). These data are in agreement with our previous results showing that Hh-Np is targeted to the apical side of the embryonic ectoderm, while Hh-N is preferentially basolaterally located (Gallet et al., 2003).

In a second set of experiments, we monitored the expression of the reporter gene *dpp-lacZ* and found that Hh-Np also induces *dpp* at a longer range than does Hh-N. Indeed, when ectopic clones of Hh-Np expressing cells were induced in the anterior compartment by the flip-out technique (see Materials and methods), *dpp* expression was observed in up to seven rows of cells around the clone (Fig. 6B-D). By contrast, only three or four rows of cells expressed *dpp* around Hh-N expressing clones of similar size (Fig. 6E-G) when clones are induced only in the columnar epithelium. In addition, *dpp* was induced in only one row of cells around clones expressing the membrane-bound Hh-CD2 (Fig. 6H-J) (Strigini and Cohen, 1997). Taken together, our results clearly demonstrate that Hh-N behaves in the wing imaginal disc as it does in the embryonic ectoderm: an absence of the cholesterol moiety on Hh impairs apical targeting and the formation of LPSs, and also restricts its range of activity.

Differential long-range activity of Hh-Np and Hh-N from peripodial cells

Burke and colleagues showed that when Hh-N is expressed under the control of the *engal4* driver in flies containing an inactivated thermo-sensitive *hh* allele, Hh-N can activate *dpp* expression at long range throughout the A compartment. This suggested that cholesterol is not necessary for the long-range activity of Hh (Burke et al., 1999).

How can we reconcile our data with these previously published data? During the course of our Hh-N flip-out clone study, we noticed that 80% of the discs displayed anterior compartment outgrowth (Fig. 7D,F). This prompted us to examine the role of the peripodial membrane, a squamous epithelium that covers the apical face of the columnar epithelium (or disc proper, Fig. 7J,K). Indeed, we

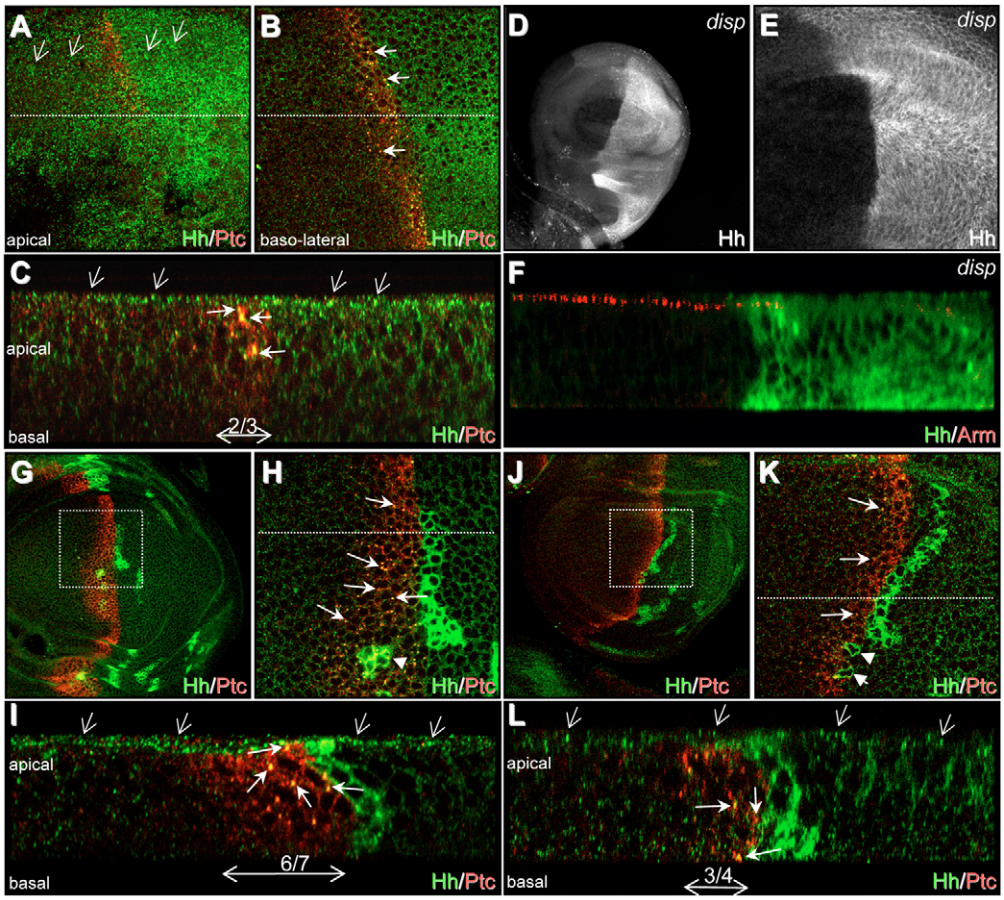

Fig. 5. Disp is required for the apical Hh-Np LPS formation in the wing imaginal disc. (A-C) Wild type, (D-F) *disp*, (G-I) *yw hs-flp; enGAL4/UAS-Hh-Np; FRT82 hh^{AC}/FRT82 tubGAL80* and (J-L) *yw hs-flp; enGAL4/UAS-Hh-N; FRT82 hh^{AC}/FRT82 tubGAL80* imaginal wing discs stained for Ptc (red in A-C,G-L) or Arm (red in F) and Hh (green). (C,F,I,L) Confocal *z*-sections corresponding to the level of the broken line in A,B,H,K. (H,K) Enlargements of the discs shown in G,J, respectively. (**A-C**) In wild-type discs, Hh-Np is secreted from the P compartment and forms apically located accretions (thin arrows in A and C) that can also be observed in anterior cells. In the first two or three rows of A-responding cells, larger intracellular accretions containing Hh and Ptc can be observed (thick arrows in B and C). (**D-F**) None of these accretions is observable in Disp mutant discs. Posterior *hh*-null clones expressing Hh-Np and abutting the AP border stimulate Ptc overexpression across a six- or seven-cell wide area (**G-I**), while clones of similar size expressing Hh-N activate Ptc in only three or four rows of cells (**J-L**). In both cases, small clones also appeared in the A compartment close to the AP boundary (arrowheads in H and K), most probably owing to *engal4* expression in this domain at late larval stages. Although in *hh^{null} UAS-hh-Np* expressing clones Hh is enriched at the apical side (I) [as is endogenous Hh-Np (C)], in *hh^{null} UAS-hh-N* expressing clones, Hh is enriched basally (L). In the latter case, we observed very basal Ptc-Hh accretions (thick arrows in L), whereas in the former case Ptc-Hh vesicles are concentrated more apically in the receiving cells (thick arrows in C,I). In all cases, Hh-Np LPSs are observable, probably owing to the surrounding wild-type cells (thin arrows in I and L).

frequently observed flip-out clones located in the peripodial membrane. Interestingly, we found that peripodial clones expressing either Hh-Np or Hh-CD2 only induced restricted *dpp* expression in columnar cells of the hinge region that underlay the peripodial clones (Fig. 7A,B,G,H,J). Translumenal cytoplasmic extensions from the peripodial membrane to the columnar epithelium have been described in the hinge and notum region (Gibson and Schubiger, 2000). Thus, it is likely that Hh-Np and Hh-N-CD2, both of which localise to the plasma membrane, activate *dpp* in underlying cells through these translumenal membrane extensions (Fig. 7J). Interestingly, both forms are unable to activate *dpp* in the underlying wing pouch, suggesting that the extensions may be incomplete or absent between squamous cells and the pouch region, as previously suggested (Gibson and Schubiger, 2000). It is also possible that the peripodial cells directly contact the hinge columnar epithelia but not the wing pouch during imaginal disc development.

In contrast to clones expressing Hh-Np or Hh-N-CD2, Hh-N expressing peripodial clones induced strong disc outgrowth, with variable levels of *dpp* expression, throughout the disc proper (Fig. 7D,E,F,K). The disc outgrowth and broad activation of *dpp* were consistent with results published by Burke et al. (Burke et al., 1999). To express Hh-N in the P columnar cells, these authors used the *engal4* driver, which drives expression not only in the columnar cells but also in the peripodial cells that cover both the A and P compartments of the disc proper (arrows in Fig. 7I). Thus, in their study, Hh-N was expressed in the peripodial cells, similar to Fig. 7D,F, and induced unrestricted activation of Hh targets within the underlying cells. Similar unrestricted activity of Hh-N has been observed (Dawber et al., 2005) that is also probably due to peripodial Hh-N-expressing cells that could have been missed because of the technical procedure used in this later study. Indeed, when flp out clones are induced at high frequency as presented in their report, in most cases clones are induced in both layers, i.e. in the peripodial and columnar cells. It is possible to miss the peripodial clones because the peripodial membrane can be easily torn off during dissection. It is also possible that the β-gal staining – which was used to label Hh-expressing cells by Dawber and colleagues – was too weak to be detectable in the thin peripodial cells.

Our results provide an explanation for these previous published data, and strongly suggest that the long-range activity of Hh-N that was described in this paper was the product of a particular experimental design that likely induced free diffusion of Hh-N from the peripodial cells into the luminal space (Fig. 7K, see Discussion).

Do these data suggest that, depending on the cell type, Hh-N has a greater range than Hh-Np? It has been shown that the secretion of Hh-Np depends on Disp activity, while that of Hh-N does not in both *Drosophila* and vertebrates (Burke et al., 1999; Gallet et al., 2003; Nakano et al., 2004; Tian et al., 2005). It is, thus, possible that

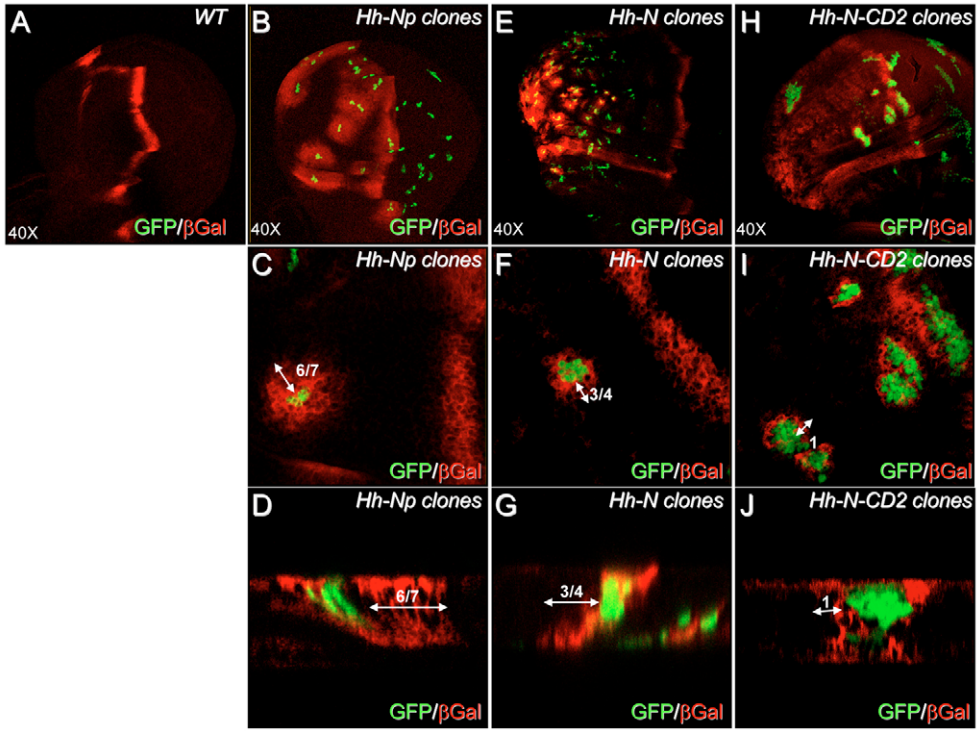

Fig. 6. Short-range activity of Hh-N in columnar cells. *dpp-lacZ* (red) expression in wild-type wing imaginal disc (**A**) or around clones of cells (marked by GFP in green) expressing either Hh-Np (**B-D**), Hh-N (**E-G**) or Hh-N-CD2 (**H-J**) produced in columnar cells using the flip-out technique. (D,G,J) Confocal *z*-sections (apical side upwards). Clones of cells expressing Hh-Np in the anterior compartment of the disc induced *dpp* expression in a six- or seven-cell wide region around the clone (B-D), while Hh-N was able only to activate *dpp* expression in a three- or four-cell region around the clone, even when the clones were bigger than Hh-Np expressing clones (E-G). Hh-N-CD2 expressing clones activated *dpp* in 1 row of cells around the clone (H-J).

peripodial cells do not have the necessary machinery for extracting Hh-Np, thereby preventing its luminal secretion. To address this possibility, we induced clones of peripodial cells expressing both Hh-Np and Disp (Fig. 7C). In this experiment, Hh-Np induced moderate levels of *dpp* expression in underlying columnar cells, accompanied by a significant outgrowth of the anterior compartment that was comparable to – or sometimes greater than – the effect observed with Hh-N (compare Fig. 7C with 7D,F). This suggests that when the peripodial cellular machinery is provided with all necessary components for Hh secretion, the activity ranges of Hh-Np and Hh-N are comparable.

DISCUSSION

Among all the metazoan proteins, Hh is the only one that has been shown to be covalently linked to a molecule of cholesterol. Particularly in view of the known function of the cholesterol group in anchoring Hh to the plasma membrane, it is important to understand what, if any, role the cholesterol adduct plays in the long-range activity of Hh. Our study of Hh activity in three different epithelia reveals that a lack of cholesterol modification on Hh impairs the controlled long-range activity of the protein.

The cholesterol adduct is necessary for full Hh activity

Several of our results indicate that the activity of Hh is lower in the Hh-N form than in the Hh-Np form. For example, in cell culture assays Hh-N displays less activity than Hh-Np in activating a reporter gene and promoting Fu and Cos-2 (data not shown) phosphorylation. Similarly, in cultured cells, Shh-N displays less activity than Shh-Np (Williams et al., 1999). On the contrary, using in vitro neural explant differentiation assays, it has been shown that Shh-N is more potent than Shh-Np, independently of its ability to be transported (Feng et al., 2004). In fact, this report suggests that the C-terminal lipid reduces the differentiation-inducing activity of Shh. Similarly, it has been shown by Lewis et al. (Lewis et al., 2001) that the short-range signalling activity present in the limb of N-Shh/Shh-null mice is comparable relative to that of wild-type Shh/+ mice, also suggesting that Shh-Np is not more potent than Shh-N in vivo.

In the wing imaginal disc, comparing Hh-N and Hh-Np autocrine activity, which is independent of Hh movement, provides evidence that Hh-N is less active. For example, autonomous induction of En can frequently be observed in ectopic Hh-Np expressing cells, whereas En is induced at a lower frequency in cells that express Hh-N (see Fig. S4 in the supplementary material). As En activation reflects the full activation of Hh signalling (Strigini and Cohen, 1997), this suggests that the absence of the cholesterol adduct prevents Hh-N from activating the Hh pathway to its highest level in vivo.

What could be the basis for this reduction in Hh activity? Because Hh-N is rendered inactive if the cysteine residue that is normally palmitoylated is altered, the reduction is probably not due to a complete absence of palmitoylation (Fig. S3) (Chamoun et al., 2001). It is also possible that the absence of cholesterol significantly reduces the efficiency of palmitate addition (Pepinsky et al., 1998) and, consequently, Hh potency. We do not favour this possibility because Chen and colleagues (Chen et al., 2004) reported that when HEK293 cells were metabolically labelled with [^{3}H]-palmitic acid, both full-length and processed Shh were labelled, as was Shh-N (without cholesterol), at quantitatively comparable levels. This indicates to us that the cleavage and cholesterylation of Shh is not a prerequisite for palmitoylation in this cell line. Moreover, reducing the proportion of Shh-N that is acylated from 80% to 30% did not induce a detectable diminution of its activity in vitro (Pepinsky et al., 1998). We also observed that expression of C85S-Hh-N, which cannot be palmitoylated, had a dominant-negative effect on Hh signalling (data not shown). In view of this, if a significant fraction of Hh-N was not palmitoylated in our transgenic animals, then we should have observed a dominant effect on Hh signalling.

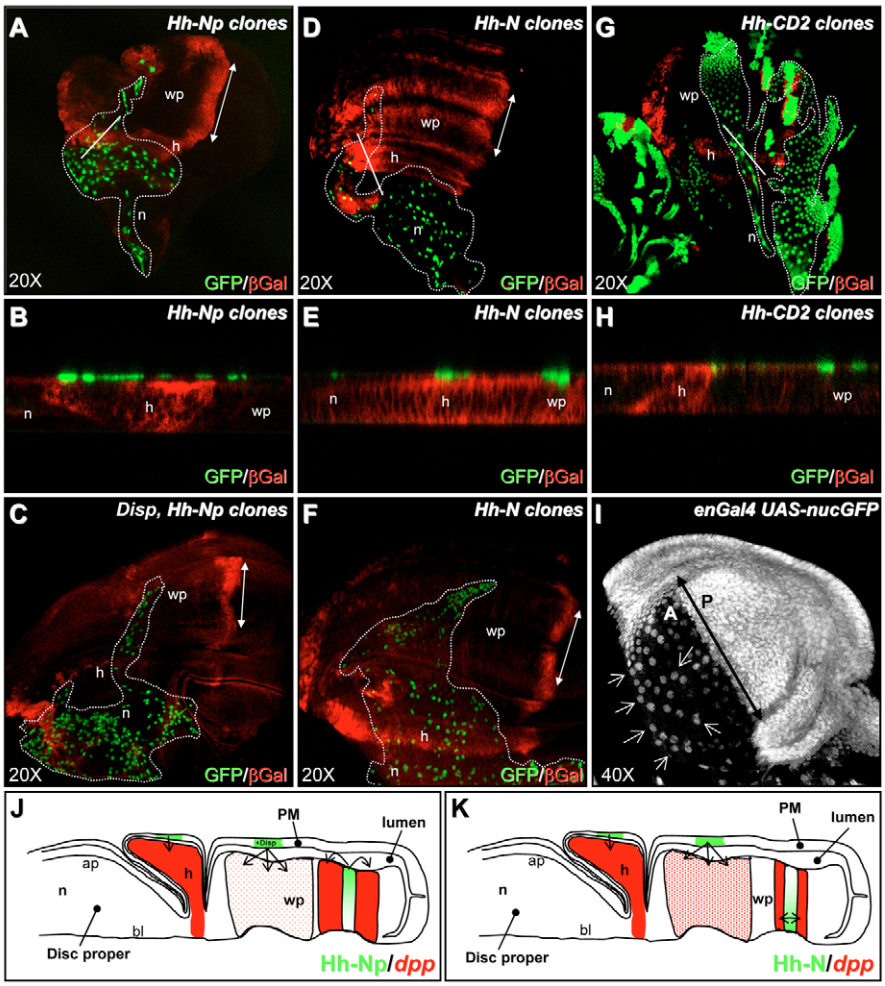

Fig. 7. Expression of Hh-N in peripodial cells induces broad activation of *dpp* expression. *dpp-lacZ* (red) expression in wing imaginal discs with clones (marked by GFP in green) of cells expressing Hh-Np (**A**,**B**), both Hh-Np and Disp (**C**), Hh-N (**D**-**F**) or Hh-CD2 (**G**,**H**) produced using the FRT flip-out technique. (B,E,H) Confocal *z*-sections (apical side upwards) made at the level indicated by the white lines in A,D,G, respectively. Peripodial clones expressing the different Hh forms are delimited by broken lines (A,D,G). (**I**) *enGal4 UAS-GFPnuc* imaginal wing disc. Peripodial clones expressing Hh-Np (A,B) or Hh-CD2 (G,H) do not trigger outgrowth of the discs, and induce *dpp* only in underlying columnar cells when the clones are located above the hinge region of the disc proper. By contrast, Hh-N clones in the peripodial membrane induce anterior compartment outgrowth and broad (in 40% of the observed discs, as in D) or moderate (in 60% of the observed discs, as in F) *dpp* expression in the disc proper in both the wing pouch and the hinge regions, although not in the notum region (D-F). Peripodial clones expressing both Hh-Np and Disp induce disc outgrowth with a moderate level of *dpp* expression in the disc proper (C). In *enGal4 UAS-GFP* discs, all the P cells and the first two rows of A cells of the disc proper are fluorescent (I) (the AP border is delimited by the double-headed arrow, based on the lower fluorescent signal observed in A cells). The large GFP-positive nuclei located in the upper layer correspond to the peripodial membrane (arrows in I). GFP-positive peripodial cells are above the A cells of the disc proper. (**J**,**K**) Schemes and model summarising the various data obtained for Hh-Np and Hh-N, respectively. Clones of Hh-Np expression in the disc proper induce widespread *dpp* expression, whereas similar Hh-N clones induce only narrow domains of *dpp* expression. In the disc proper, Hh-Np is enriched apically (ap), whereas Hh-N is enriched at the basolateral (bl) membrane. By contrast, clones of Hh-N in the peripodial membrane (PM) allow luminal diffusion of Hh-N, inducing widespread *dpp* expression in the wing pouch and the hinge. Peripodial Hh-Np clones induce *dpp* in the hinge through cellular interactions, with no apparent consequences for the wing pouch. When both Disp and Hh-Np are present in peripodial cells, luminal secretion of Hh-Np is permitted, resulting in outgrowth and moderate levels of *dpp* expression in the wing pouch (hatched area). n, notum; h, hinge; wp, wing pouch.

Instead, the decreased Hh-N activity is most likely caused by the lack of cholesterol modification. Both in vitro and in vivo data suggest that the absence of cholesterol decreases the association of Hh-N with the membrane on receiving cells (Fig. 1B′; Fig. 3D). Thus, one possible explanation for the reduced activity of Hh-N in S2 cells and in autocrine assay is that it interacts less efficiently with the plasma membrane of receiving cells. This conforms to previous data showing that cholesterol promotes the stable membrane association of lipid-modified proteins (Peters et al., 2004). A consequence of this might be that Hh-N has less time to activate Hh receptors, as it diffuses away from the cell membrane in three dimensions rather than two, lowering its apparent intrinsic activity.

Cholesterol is required for controlled Hh long-range activity

In addition to their apparent activity differences, our data also suggest that the two forms of Hh differ in their ranges of action. Reducing the movement of Hh through the expression of a membrane-tethered form, Hh-N-CD2, revealed that Hh movement is required for specifying certain cell types such as distant type 3 cells within the dorsal ectoderm, for the repression of distant *ser* expression in the ventral ectoderm, and for the expression of *dpp* and *ptc* in distant imaginal disc cells. Similarly, removing the cholesterol adduct restricts the long-range activity of Hh (Figs 1, 2, 5 and 6), except when Hh-N is expressed in the peripodial cells (Fig. 7; see below).

If cholesterol were necessary only for potentiating Hh activity, then those target domains requiring the highest levels of Hh should be reduced in Hh-N-expressing animals, and those specified by lower Hh levels should be increased. Interestingly, we obtained the opposite results when Hh-N was expressed. For example, distant dorsal type 3 cells, which require low Hh levels, were absent in Hh-N expressing embryos (Fig. 2E,J-K). This suggests that the absence of cholesterol modification limits the range of activity of Hh-N. Moreover, in columnar cells, Hh-N induced a restricted domain of *dpp* expression, which is a marker of low Hh-signalling activity, in contrast to the broader *dpp* domain induced when Hh-Np is expressed. This result was not caused by a decrease in the intrinsic activity of Hh-N, because when Hh-N was expressed in peripodial cells it activated *dpp* in

all of the underlying cells of the disc proper (Fig. 7D). This provides strong evidence that it retains enough substantial activity to activate the pathway.

The unrestricted range of Hh-N activity observed in this latter experiment allows us to propose that the expression of Hh-N in the squamous peripodial cells permits it to enter the lumen and spread unrestrictedly, probably through free diffusion within this extracellular space. The accumulation of Hh-N, probably at the apical side of receiving cells, reaches the threshold necessary for *dpp* activation and wing disc outgrowth. This is clearly distinct from what happens when Hh-N is expressed in the disc proper, where it is basally located (Fig. 5L) and restricted in its range of activity, consistent with it being dispersed through the basal membrane of the epithelium and the haemolymph. In contrast to Hh-N, Hh-Np is apically enriched in both columnar and ectodermal cells (Fig. 5C,I) (Gallet et al., 2003), and acts at a longer distance (Figs 2, 5 and 6), although not by spreading through the imaginal disc lumen (Fig. 7A). Rather, its movement is consistent with it being restricted to a two-dimensional space. This correlates with data showing that HSPGs are required for the stable retention of Hh-Np on the cell surface (Gorfinkiel et al., 2005; Takei et al., 2004), suggesting a mechanism by which the movement of Hh-Np could be maintained within a continuous plane. Because HSPGs are not involved in Hh-N spreading (The et al., 1999), it is thus possible that when Hh-N is expressed in the different epithelia, its dispersal within the three-dimensional space rapidly lowers its local concentration, explaining why its long-range activity is affected but its short-range activity is not.

Why is Hh-Np not secreted into the disc lumen in the same way as Hh-N is when expressed in peripodial cells? Our data suggest that Disp activity is absent from these cells (Fig. 7C). Indeed, it is only when both Hh-Np and Disp are co-expressed in peripodial cells that long-range activity is manifested by an important wing pouch outgrowth. This is the only experimental setting in which the activity ranges of Hh-N and Hh-Np are similar (Fig. 7). Nevertheless, this similarity is unusual because free dispersal of Hh in the lumen between peripodial and columnar cells is unlikely under physiological conditions. Endogenous expression of Hh in peripodial cells has been implicated in leg disc regeneration (Gibson and Schubiger, 1999). Our data support the hypothesis that endogenous peripodial Hh does not influence underlying columnar cells in wild-type animals, probably because it is not secreted.

Functional Hedgehog gradient

Our data show that the differential long-range activity of the two forms depends on differences in Hh routing in producing epithelial cells. This underlines the importance of the apical site of secretion as a prerequisite for the controlled planar movement of Hh-Np in various tissues (Fig. 7J,K). Several lines of evidence suggest that Hh-Np spreads via a planar movement on the apical epithelial cell surface, and not basally through transcytosis, which involves internalisation and secretion on a cell-by-cell basis. First, an absence of dynamin, which is required for internalisation, prevents neither Hh-spreading nor its long-range activity (Gallet and Therond, 2005; Han et al., 2004b; Torroja et al., 2004). Second, in the absence of the Ptc receptor, Hh-Np is stuck at the apical surface of receiving cells (Gallet and Thérond, 2005). Third, after internalisation, the Hh-Ptc complex is targeted to the lysosomal compartment (Gallet and Therond, 2005; Incardona et al., 2002; Torroja et al., 2004) and is not recycled, at least not through a Rab11-dependent mechanism (data not shown). Interestingly, Han and colleagues reported that Hh accumulates at both the apical and basolateral sides of receiving cells that were impaired for dynamin activity (Han et al., 2004b). We favour the model in which the functional Hh gradient spreads apically through the epithelia, but we cannot exclude a contribution of a transcytosis-independent basolateral functional gradient of Hh, at least for short-range signalling. The two models are not exclusive.

Importance of Hh assembly into LPS for long-range activity

The cholesterol-dependent formation of LPSs might be an important vehicle in the establishment of long-range Hh activity. We show here that Hh-Np forms LPSs, which can be observed at a distance from the Hh source in the disc (see Gallet et al., 2003). We also show that Hh-Np is able to dimerise and is present in fractions that suggest a multimerisation of the molecule. By contrast, Hh-N is unable to form LPSs, behaves as a monomer and acts at shorter range. We suggest that Hh-Np multimerisation allows maximal long-range signalling of Hh. However, we cannot exclude the possibility that monomeric Hh-Np is involved in short-range signalling.

The oligomerisation of Hh-Np could be necessary for this highly hydrophobic molecule to travel within hydrophilic environments by hiding its hydrophobic domains within micelle-like structures, as suggested for Shh-Np (Feng et al., 2004; Zeng et al., 2001). These structures could create a specific interaction with HSPG at the surface of the cell membrane. Indeed, Dly has been shown to specifically colocalise with Hh-Np (Han et al., 2004b), and it has been shown that HSPGs regulate Hh-Np movement as well as its stability (Lin, 2004). It is thus possible that the movement of apical Hh multimers/LPSs depend on HSPG enriched at the apical side of the cells. Thus, Hh-LPSs might not only provide a vehicle for Hh, but may also allow the planar movement of Hh in two dimensions, thereby avoiding a dilution of Hh-Np activity in the disc lumen.

In conclusion, our data in *Drosophila* strongly suggest that absence of cholesterol-modification in Hh-Np affects its secretion, multimerisation and long-range signalling activity. Our data reconcile evidence from mammalian systems regarding the importance of cholesterol in long-range Hh activity with previous results from *Drosophila* (Burke et al., 1999; Dawber et al., 2005; Lewis et al., 2001).

We are grateful to K. Basler, M. Gonzalez-Gaitan, I. Thé, M. van den Heuvel, J. Pradel and S. Cohen for fly stocks. We especially thank Ralph Rodriguez for providing Fig. 1D,E and for his help in collecting embryos. We also thank all 'fly' members of the ISDBCR for exciting discussions about this work. This work was supported by grants from ARC, Ligue Nationale Contre le Cancer and the ATIPE program of the Centre National de la Recherche Scientifique to P.P.T.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/3/407/DC1

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