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Dispatched, a Novel Sterol-Sensing Domain Protein Dedicated to the Release of Cholesterol-Modified Hedgehog from Signaling Cells

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Summary

Members of the Hedgehog (Hh) family of secreted signaling proteins function as potent short-range organizers in animal development. Their range of action is limited by a C-terminal cholesterol tether and the upregulation of Patched (Ptc) receptor levels. Here we identify a novel segment-polarity gene in *Drosophila*, *dispatched* (*disp*), and demonstrate that its product is required in sending cells for normal Hh function. In the absence of Disp, cholesterol-modified but not cholesterol-free Hh is retained in these cells, indicating that Disp functions to release cholesterol-anchored Hh. Despite their opposite roles, Disp and Ptc share structural homology in the form of a sterol-sensing domain, suggesting that release and sequestration of cholesterol-modified Hh may be based on related molecular pathways.

Introduction

Throughout the animal kingdom, morphogenetic signaling molecules are used to inform cells of their relative position within developing tissues. Major emphasis has been devoted in the past to demonstrating that such signaling molecules can act over large distances and directly influence the fate of cells far away from their site of synthesis. Compelling evidence has recently been obtained that shows that members of the TGF-β, Wnt, and Hedgehog (Hh) families signal over many cell diameters in a direct and concentration-dependent manner to organize tissue pattern (Lawrence and Struhl, 1996; Hammerschmidt et al., 1997; Neumann and Cohen, 1997). It becomes increasingly clear, however, that in many instances the range of a signaling molecule should not exceed a certain distance, and its movement must therefore be subject to tight regulation.

This is particularly well documented in the case of *Drosophila* limb development, where the short-range inducer Hh controls the expression of the long-range morphogens Wingless (Wg) and Decapentaplegic (Dpp, Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Zecca et al., 1995). Each leg and wing primordium is subdivided into two cell populations, the anterior (A) and posterior (P) compartments. Cells in the P compartment are programmed by the selector gene *engrailed* (*en*) to secrete Hh. *en* is not active in A compartment cells, and as a consequence, these cells are competent to transduce the Hh signal (reviewed by Lawrence and Struhl, 1996). Essential to correct wing patterning is the restriction of Hh signaling activity to a narrow band of A cells. Firstly, the range of Hh signaling directly determines the positioning of the future wing vein L3 (Mullor et al., 1997; Strigini and Cohen, 1997). Secondly, Hh induces a narrow stripe of cells to express *dpp*. Dpp then acts as a long-range morphogen to induce more distant pattern elements along the entire anteroposterior axis (Lecuit et al., 1996; Nellen et al., 1996). When the spatial extent of *dpp* expression is altered, patterning is dramatically affected (Zecca et al., 1995). The short-range nature of Hh signaling appears to rely on at least two unprecedented mechanisms of receptor circuitry and ligand biosynthesis.

The Hh signal is transduced by a receptor complex consisting of the two cell surface proteins, Patched (Ptc) and Smoothened (Smo, reviewed by Ingham, 1998). Ptc is expressed in all A compartment cells and, in the absence of Hh, inhibits the activity of Smo, which is essential for Hh signal transduction. Binding of Hh to Ptc releases latent Smo activity, activating the transduction pathway (Chen and Struhl, 1998; Murone et al., 1999). A universal response to the Hh signal in all systems examined so far is the upregulation of *ptc* transcription (Forbes et al., 1993; Goodrich et al., 1996; Marigo et al., 1996). In an elegant series of experiments, it was shown that the accumulation of Ptc protein in Hh-responding wing cells sequesters Hh protein and thereby restricts the further movement of Hh into the A compartment (Chen and Struhl, 1996).

The second mechanism by which Hh movement is impeded has been elucidated in detailed biochemical studies investigating the processing of Hh to its active signaling form. Hh undergoes an autoproteolytic cleavage reaction to give rise to its active N-terminal portion (Lee et al., 1994; Porter et al., 1995). This cleavage is accompanied by the covalent bonding of a cholesterol moiety to the C terminus of this N-terminal portion, producing the active Hh, termed Hh-Np ("p" standing for "processed", see Porter et al., 1996b). Hh protein derived from a transgene that encodes only the N-terminal portion does not undergo cleavage and is consequently not linked to cholesterol. When this unmodified protein, Hh-Nu ("u" standing for "unmodified"), is expressed in the *Drosophila* embryo, a broader range of Hh action than normal is observed (Porter et al., 1996a). One interpretation of these results is that Hh-Nu can move further than Hh-Np due to the absence of the cholesterol modification. Intriguingly, Ptc contains a sterol-sensing domain (SSD, reviewed by Osborne and Rosenfeld, 1998),

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which has been shown in proteins such as HMG CoA reductase (Gil et al., 1985) and SREBP cleavage-activating protein (SCAP, Hua et al., 1996) to be able to monitor sterol levels in membranes. One possibility is that Ptc interacts directly with the cholesterol moiety of Hh-Np via its SSD, thus sequestering Hh and restricting its motility (Beachy et al., 1997).

While the cholesterol adduct appears to be necessary for the restriction of Hh motility, its presence poses a severe dilemma for Hh-Np. Such lipid modifications act as anchors, tethering proteins at membrane surfaces. Thus, a central, unresolved question is how Hh-Np, which is presumably produced in the endoplasmic reticulum (ER), is released from Hh-producing cells such that it can move to cells at a distance.

Here we provide evidence that an active process is required to release cholesterol-modified Hh-Np from producing cells. We describe the identification of a novel 12-pass transmembrane protein, Dispatched (Disp), with sequence similarity to vertebrate and *Drosophila* Ptc (Hooper and Scott, 1989; Nakano et al., 1989; Goodrich et al., 1996; Hahn et al., 1996) and Niemann-Pick Type C (NPC1, Carstea et al., 1997; Loftus et al., 1997) proteins. Disp is required only in P cells for effective Hh signaling to A cells. In the absence of Disp, Hh is retained in P cells, and its access to A cells is severely limited. In contrast, Hh-Nu is unaffected by the absence of Disp, indicating that the cholesterol anchor is responsible for the retention of Hh in *disp* mutant cells. Our results indicate that Hh-Np is indeed a tethered protein but that its retention is overcome by the activity of the dedicated SSD protein, Disp.

Results

Identification of *dispatched*, a Novel Gene Required for Hh Signaling

In a genetic screen for novel components of the *Drosophila* Hh signaling pathway, we identified a mutation on the third chromosome causing phenotypes typical of those resulting from loss of *hh* or *wg* function. Animals zygotically homozygous for this mutation survive until early pupal stages. However, animals lacking in addition the maternal component of this locus die during embryogenesis with a strong segment-polarity phenotype. Instead of the wild-type segmentally repeated pattern of denticle belts interspersed by naked cuticle (Figure 1A), such embryos display a lawn of denticle belts and fail to secrete naked ventral cuticle (Figure 1B). Germline clone-derived embryos are rescued by a wild-type paternal chromosome, indicating that the gene product is required only after the onset of zygotic transcription. Segment-polarity phenotypes are indicative of loss-of-function mutations in essential components of the Hh (Figure 1C) and Wg signal transduction pathways (Nüsslein-Volhard and Wieschaus, 1980). Due to its presumed role and the structural similarities and functional dissimilarities to *ptc* described below, we have named this novel gene *dispatched* (*disp*).

To determine if *disp* is required specifically for either the Hh or Wg pathway, we generated large $disp^{-/-}$ clones in the adult wing, a tissue in which the two pathways function independently of each other in distinct subpopulations of cells. Loss of Wg signaling in the wing primordium results in loss of wing margin (Couso et al., 1994), whereas a reduction in Hh activity causes a strong narrowing of the intervein region between longitudinal veins L3 and L4 (Slusarski et al., 1995; Sanchez-Herrero et al., 1996; Méthot and Basler, 1999). Although $disp^{-/-}$ clones can encompass large regions of Wg-sending and Wg-receiving cells, they contribute to wild-type wing margin structures (Figure 1D), which indicates that *disp* function is not required for the Wg signaling pathway. However, when located in the posterior compartment, large clones cause a significant reduction in the distance between veins L3 and L4 (Figure 1E), a phenotype typical for the reduction of Hh signaling at the A/P boundary (Figure 1F). Thus, we conclude that *disp* is acting in the Hh signaling pathway.

disp Encodes a Putative Multipass Transmembrane Protein with an SSD

We mapped the *disp* gene to cytological position 83C, cloned genomic sequences of *disp*, and isolated corresponding cDNA clones (see Experimental Procedures). The composite sequence from these cDNAs revealed an open reading frame (ORF) encoding a putative protein of 1218 amino acids (Figure 2A). A transgene containing the full-length ORF driven by the weak, ubiquitous promoter of the *tubulin*α*1* gene was introduced into the *Drosophila* germline and fully rescued $disp^{-/-}$ animals to viable adults, confirming that the cloned gene is indeed responsible for the pupal lethality and wing phenotype caused by the *disp* mutation. In addition, rescued animals are fully fertile when crossed *inter se*, indicating that the transgene also rescues the embryonic segment-polarity phenotype associated with the absence of *disp* function.

Searches of genome databases revealed structural homologies of the Disp protein to the products of the vertebrate *ptc* (Goodrich et al., 1996; Hahn et al., 1996; Marigo et al., 1996) and *NPC1* (Carstea et al., 1997; Loftus et al., 1997) disease genes and their *Drosophila* homologs (Figure 2B, Hooper and Scott, 1989; Nakano et al., 1989). Based on the TopPred 2 (von Heijne, 1992) transmembrane domain prediction algorithm, Disp contains 12 putative membrane-spanning domains (Figure 2A). Like the Ptc and NPC1 proteins, Disp has a sterol-sensing domain (SSD, Figure 2A), a domain first defined in HMG CoA reductase (Gil et al., 1985) and SCAP (Hua et al., 1996). These two proteins are key regulators of intracellular cholesterol homeostasis, while NPC1 is thought to be involved in cholesterol trafficking, since defects in this protein cause an accumulation of cholesterol in lysosomes (reviewed by Liscum and Klansek, 1998). Aside from the multitransmembrane domain structure and the SSD, no other homologies to Ptc or NPC1 proteins could be detected in Disp. The protein with the highest overall homology to Disp is the product of an as yet uncharacterized *C. elegans* gene (GenBank acc. no. AAC48001, here termed ceDisp). We propose that together Disp and ceDisp define a novel subfamily of SSD proteins.

To determine the expression pattern of *disp*, we probed wild-type embryos and imaginal discs with DIG-labeled sense and anti-sense *disp* RNA. While no staining was observed using the sense strand (Figures 2C′

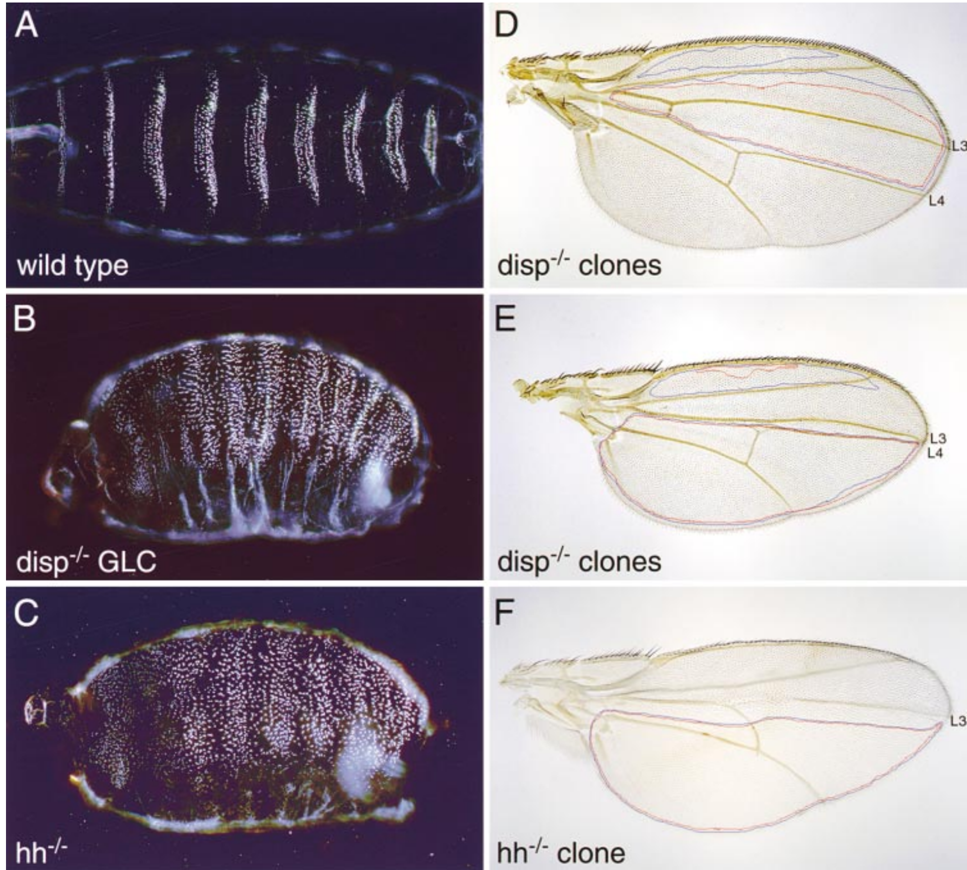

Figure 1. Loss of *disp* Function Causes *hh*-like Phenotypes in the Embryo and the Adult Wing

(A–C) Cuticle preparations of late stage embryos. Ventral view. Embryos are oriented anterior to the left. (A) Wild-type embryo. (B) $disp^{-/-}$ embryo derived from a mutant germ line clone. (C) $hh^{AC/AC}$ homozygous mutant embryo.

(D–F) Adult wings with genetically marked mutant clones. Wings are oriented proximal to the left, anterior up. Mutant clones are outlined: red denotes dorsal wing surface, blue denotes ventral surface. (D) Large $disp^{-/-}$ clones in the A compartment. Wing patterning and size is unaffected. (E) Large $disp^{-/-}$ clone in the P compartment. The intervein region between longitudinal veins L3 and L4 is strongly reduced. The wing is reduced in size, but otherwise patterning remains normal. An additional far anterior clone shows no phenotypic effect. (F) Large $hh^{AC/AC}$ clone in the P compartment. The phenotype is similar to that caused by *disp* mutant clones, although vein L4 is additionally disrupted.

and 2D′), ubiquitous *disp* expression was observed throughout the embryo (Figure 2C) and imaginal discs (Figure 2D) when the anti-sense strand was used. Thus, based on its expression pattern, *disp* is neither a transcriptional target nor a spatial determinant of Hh signaling.

Disp Is Required in Hh-Secreting but Not Hh-Receiving Cells

The adult wing clones suggested a requirement for *disp* function in P compartment cells. To confirm and extend this finding, we assayed the effect of *disp* mutant clones on Hh signaling in the wing imaginal disc, where the A/P boundary can be precisely defined and where the transcription of the Hh target genes *ptc* and *dpp* serve as immediate readouts of Hh signaling activity. We found that even large clones of $disp^{-/-}$ A cells abutting the A/P boundary had no discernible effect on *ptc-lacZ* (Figure 3A) or *dpp-lacZ* expression (not shown). Thus, despite its Ptc-like structure, Disp plays no role in transducing the Hh signal in responding A cells. In contrast, large clones of $disp^{-/-}$ P cells abutting the A/P boundary caused a dramatic reduction of both *ptc-lacZ* (Figure 3B) and *dpp-lacZ* expression (not shown). As this requirement is very similar to that for *hh* itself (Figures 3C and 3D), we interpret this result as evidence that Disp is essential for the effective production of the Hh signal in P cells. Even a small patch of $disp^{+/-}$ cells at the A/P boundary was sufficient to locally rescue Hh signaling (Figure 3E), impressively demonstrating the potency of the Hh signal and its requirement for *disp* activity. One obvious explanation for the phenotypes associated with *disp* mutant cells would be an involvement of Disp in the expression of the *hh* gene itself. However, we find that *hh-lacZ* expression is unaffected in $disp^{-/-}$ clones (Figure 3F), which rules out a requirement for Disp in *hh* transcription.

Disp Is a Protein Dedicated to Hh Signaling

The experiments described above show that Disp is necessary for Hh signaling in Hh-producing cells. They do not address, however, whether the ubiquitously expressed Disp protein plays a role in other signaling pathways or in physiological processes. To investigate this

A

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MLCFDSERMNWYYHVLARRPYLVVVSIAVYCVACIIVALVLNKLPDFSDPTLGFETRGTK
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SSD

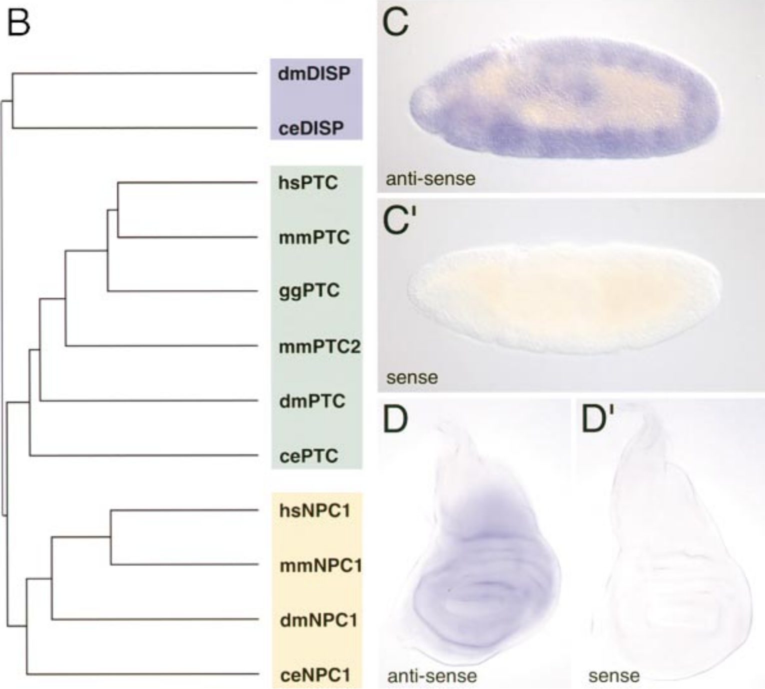

Figure 2. ***dispatched*** **Encodes a Putative 12-Pass Transmembrane Protein with Homology to** ***ptc*** **and** ***NPC1*** **Genes**

(A) Amino acid sequence of the Disp protein. Predicted transmembrane domains ("TopPred 2," von Heijne, 1992) are highlighted in gray. The putative sterol-sensing domain (SSD) is shaded in red. The GenBank accession number for Disp is AF200691.

(B) Evolutionary tree produced by "GCG Pile Up," comparing the ***Drosophila*** **Disp protein to various vertebrate and** ***Drosophila*** **Ptc and NPC1 proteins. Disp is only distantly related to Ptc and NPC1. Its closest homolog is an as yet uncharacterized** ***C. elegans*** **protein (GenBank acc. no. AAC48001), here named ceDisp. Homology is highest at the SSD, where the degree of resemblance (% identity / % similarity) of this region of Disp with the comparable region of dmNPC1, mmNPC1, mmPtc, and dmPtc is 25/47, 26/46, 26/46, and 21/43, respectively. The abbreviations used are as follows: dm,** ***Drosophila melanogaster*****; ce,** ***Caenorhabditis elegans*****; hs,** ***Homo sapiens*****; mm,** ***Mus musculus*****; gg,** ***Gallus gallus*****.**

(C and D) mRNA expression pattern of ***disp*** **in the** ***Drosophila*** **embryo (C) and wing imaginal disc (D). Staining in both cases is essentially ubiquitous, compared to the "sense" controls (C′ and D′) where no staining is observed.**

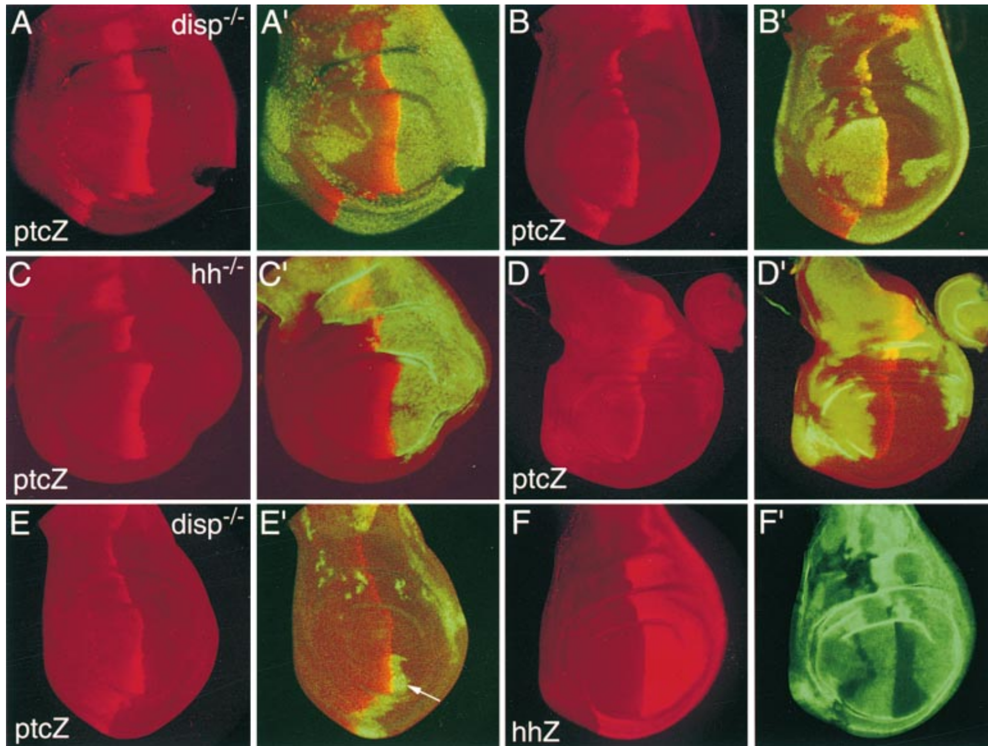

Figure 3. *disp* Is Required Only in Posterior (P) Cells for Correct Expression of the Hh Target Gene *ptc* in Anterior (A) Cells

Third instar wing imaginal discs containing *disp*$^{-/-}$ clones in a *Minute* (A, B, and E) or wild-type (F) genetic background, and *hh*$^{-/-}$ clones in a *Minute* background (C and D). In this and following figures, anterior is to the left, dorsal up. Mutant clones are marked by the absence of the πMyc (A–E), or CD2 (F) marker, shown in green. *LacZ* reporter gene expression is shown in red. In (A)–(E), the first panel shows *lacZ* expression alone, and the second panel shows a merge of the *lacZ* expression and the clone marker.

(A) *disp*$^{-/-}$ clones in the A compartment have no effect on *ptc-lacZ* expression.

(B) Large *disp*$^{-/-}$ clones in the P compartment strongly reduce the width of the *ptc-lacZ* stripe in neighboring anterior cells. Small clones along the boundary do not show this effect.

(C and D) *hh*$^{-/-}$ clones have the same effect as *disp*$^{-/-}$ clones; only large P clones (D) reduce *ptc-lacZ* expression, not A clones (C).

(E) A small patch of *disp*$^{+/-}$ cells in the P compartment (arrow) is sufficient to restore *ptc-lacZ* expression in neighboring anterior cells.

(F) *disp*$^{-/-}$ clones in the P compartment have no effect on *hh-lacZ* expression. The first panel shows *hh-lacZ* expression; the second panel shows the clone marker alone.

issue, we generated animals in which *disp* expression was restricted to Hh-secreting cells. This was achieved by introducing a *UAS-disp* transgene together with a P cell–specific *en-Gal4* driver into a *disp* mutant background. The *en-Gal4* driver is inactive in A compartment cells, which do not secrete Hh but comprise approximately two thirds of the embryonic, larval, and adult animal. *en-Gal4* is also not active in eye imaginal disc cells, which do, however, secrete Hh. The *en-Gal4 UAS-disp* transgene combination rescued *disp* mutant animals to adulthood. The resulting flies displayed normal patterning in the wing, leg, notum, and abdomen (not shown), and gave rise to viable offspring, which demonstrates that in larval and embryonic tissues, *disp* function is only required in Hh-producing cells. These rescued animals showed, however, a dramatic reduction in eye size (not shown), which indicates that Disp is also required for Hh signaling in the eye, and that the rescue observed in other tissues is due solely to *disp*$^{+}$ transcripts provided by the *en-Gal4* driver. Importantly, throughout all stages of development, A compartment cells develop and differentiate normally and become correctly patterned by numerous signaling molecules other than Hh in the complete absence of functional Disp protein. Thus, despite its ubiquitous expression, Disp is required exclusively for Hh signaling, and not for other known signaling pathways, nor for sterol homeostasis or membrane integrity.

Hh Processing Occurs Normally in *disp* Mutant Cells

As *disp* is required in Hh-producing cells for Hh signaling, but not for *hh* transcription, we examined whether Disp may be required for the processing of Hh into the active signaling moiety, Hh-Np. This processing event involves the autocatalytic cleavage of full-length Hh precursor protein to the N-terminal portion Hh-N (Lee et al., 1994; Porter et al., 1995), with the concomitant covalent linkage of cholesterol to the C-terminal amino acid to form Hh-Np (Porter et al., 1996b). We assayed this cleavage event by Western blot analysis. Transgenes encoding either full-length *hh* cDNA (*hh-F*^{HA}) or only the N-terminal portion of Hh (*hh-N*^{HA}) were expressed under *en-Gal4* control in imaginal discs. Each of these constructs was tagged with an HA epitope just N-terminal

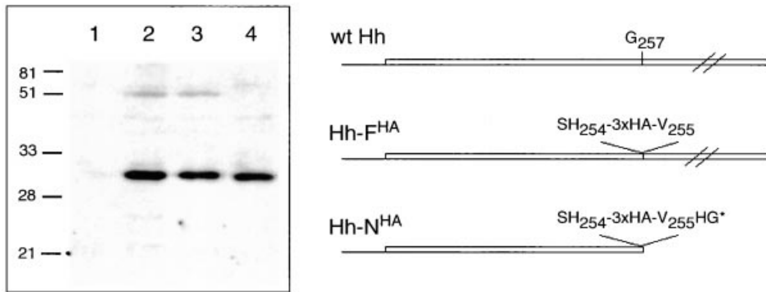

Figure 4. *disp* Is Not Required for Hh Cleavage

Western blot probed with α-HA antibody directed against HA-tagged Hh proteins. The transgenes used in this and subsequent experiments, *hh-F^HA^* and *hh-N^HA^*, are shown schematically on the right, compared to wild-type *hh*. Both have a 3× HA tag inserted just prior to the autocatalytic cleavage site (G257). The expression of both constructs induces ectopic *dpp-lacZ* expression in A compartment cells (not shown), which demonstrates that they are active in vivo. Protein extracts were derived from third instar larvae. Molecular weight markers are shown on the left. Lane 1: wild-type larvae. Lane 2: wild-type larvae expressing *hh-F^HA^*. Lane 3: $disp^{-/-}$ larvae expressing *hh-F^HA^*. Lane 4: wild-type larvae expressing *hh-NHA*. The major bands (30 kDa) in lanes (2)–(4) correspond to the Hh autocatalytic cleavage product. The minor bands in lanes (2) and (3) correspond to full-length Hh. In this and several other Western blots using the same protein sources, the ratio of full-length to cleaved Hh is unaltered in *disp* mutant animals (compare lanes (2) and (3)). The Hh-N bands seen are significantly larger than those predicted, suggesting that the N-terminal signal sequence is not removed efficiently from the construct-derived proteins.

to the defined cleavage site to allow protein detection with an α-HA antibody.

In lysates of wild-type larvae expressing $hh\text{-}F^{HA}$, two prominent bands of ~50 kDa and ~30 kDa were observed (Figure 4A, lane 2) that are absent in lysates from control animals (Figure 4A, lane 1). These two proteins correspond to unprocessed full-length Hh and processed Hh-Np, respectively. In lysates from animals expressing tagged $hh\text{-}N^{HA}$, only a single major protein species of ~30 kDa was detected (Figure 4A, lane 4) that comigrates with the smaller protein seen from animals expressing $hh\text{-}F^{HA}$, confirming that this smaller band is the result of internal cleavage of the $hh\text{-}F^{HA}$ product. When $hh\text{-}F^{HA}$ was expressed in *disp* mutant animals, the same ratio of full-length Hh to Hh-Np was observed (Figure 4A, lane 3 compared with lane 2), indicating that Hh cleavage is occurring at the same efficiency in *disp* mutant cells. From this result we conclude that the defect in Hh signaling imposed by the lack of Disp is not due to faulty cleavage of the Hh precursor protein. We failed to achieve conditions under which the status of cholesterol modification could be assessed. However, since the covalent addition of cholesterol is coupled to the cleavage reaction, which occurs normally in *disp* mutant cells, we assume that Hh-N is properly modified in the absence of Disp. In support of this assumption it should be noted that Hh lacking a C-terminal cholesterol moiety would produce an increased, rather than a decreased, spatial response to Hh. Hence, we dismiss the possibility that Disp is required for cleavage and cholesterol modification of Hh.

Retention of Hh in *disp* Mutant Cells

We next investigated whether the distribution of Hh protein is altered in the absence of Disp. As observed previously by others (Tabata and Kornberg, 1994), wild-type Hh protein normally accumulates in intracellular punctate structures in A cells near the A/P border (Figure 5A). These accumulations of Hh antigen colocalize with punctate Ptc staining (Figure 5A), suggesting they might reflect vesicular signaling complexes. When $disp^{-/-}$ discs were stained with Hh antisera, no Hh staining at all was observed in A cells (Figure 5B), whereas staining in P cells was significantly higher than in wild-type discs. To confirm this increase in Hh levels, we generated marked $disp^{-/-}$ clones and analyzed the distribution of Hh antigen in single discs. Strong accumulation of Hh levels in $disp^{-/-}$ P cells was observed in comparison to neighboring wild-type P cells (Figure 5C). Together, these results indicate that in the absence of Disp, Hh is predominantly retained in producing cells and is thus unable to move in significant quantities to A cells. Since some weak Ptc expression is still observed in A cells of *disp* mutant discs, a small fraction of Hh protein must be escaping, but in vastly reduced quantities below the limits of detection.

We then asked whether the retention of Hh in *disp* mutant tissue might reflect defects in the intracellular trafficking of Hh protein. This possibility was raised by the observation that in embryonic epidermal cells, Hh-Nu is mainly apical while Hh-Hp is predominantly basolateral, which suggests a role for the cholesterol modification in sorting (Taylor et al., 1993; Tabata and Kornberg, 1994; Porter et al., 1996a). Using Hh antisera, we could not detect any specific localization along the apical/basal axis of wing imaginal disc cells, and we did not observe an alteration in Hh distribution in $disp^{-/-}$ compared to wild-type tissue (Figures 5D and 5E). To examine whether the different isoforms might nevertheless be differently distributed in *disp* mutant cells, we then examined the surface distribution of $Hh\text{-}F^{HA}$ and $Hh\text{-}N^{HA}$ in wild-type and *disp* mutant tissue. In these experiments, the antibody was applied prior to fixation and permeabilization in order to visualize only cell surface antigen. In both wild-type and *disp* mutant tissues, $Hh\text{-}F^{HA}$ was detected on both the basal (Figure 5F) and apical (not shown) surfaces, while $Hh\text{-}N^{HA}$ was exclusively apical (Figures 5G and 5H). Due to the difficulty in accurately quantitating levels of cell surface staining, we were unable to determine if the accumulation of Hh seen within *disp* mutant cells also occurs at the cell surface. We conclude, however, that Hh is still able to reach the surface of cells lacking Disp, and although we can not rule out that Disp is required to differentially sort some small, active fraction of total Hh protein, our results argue against a role of Disp in apical/basal sorting of Hh.

The Cholesterol Anchor of Hh-Np Is Responsible for Retention of Hh in the Absence of Disp

One candidate effector for the retention of Hh in *disp* mutant cells is the cholesterol moiety, which could conceivably tether Hh to the membranes of producing cells.

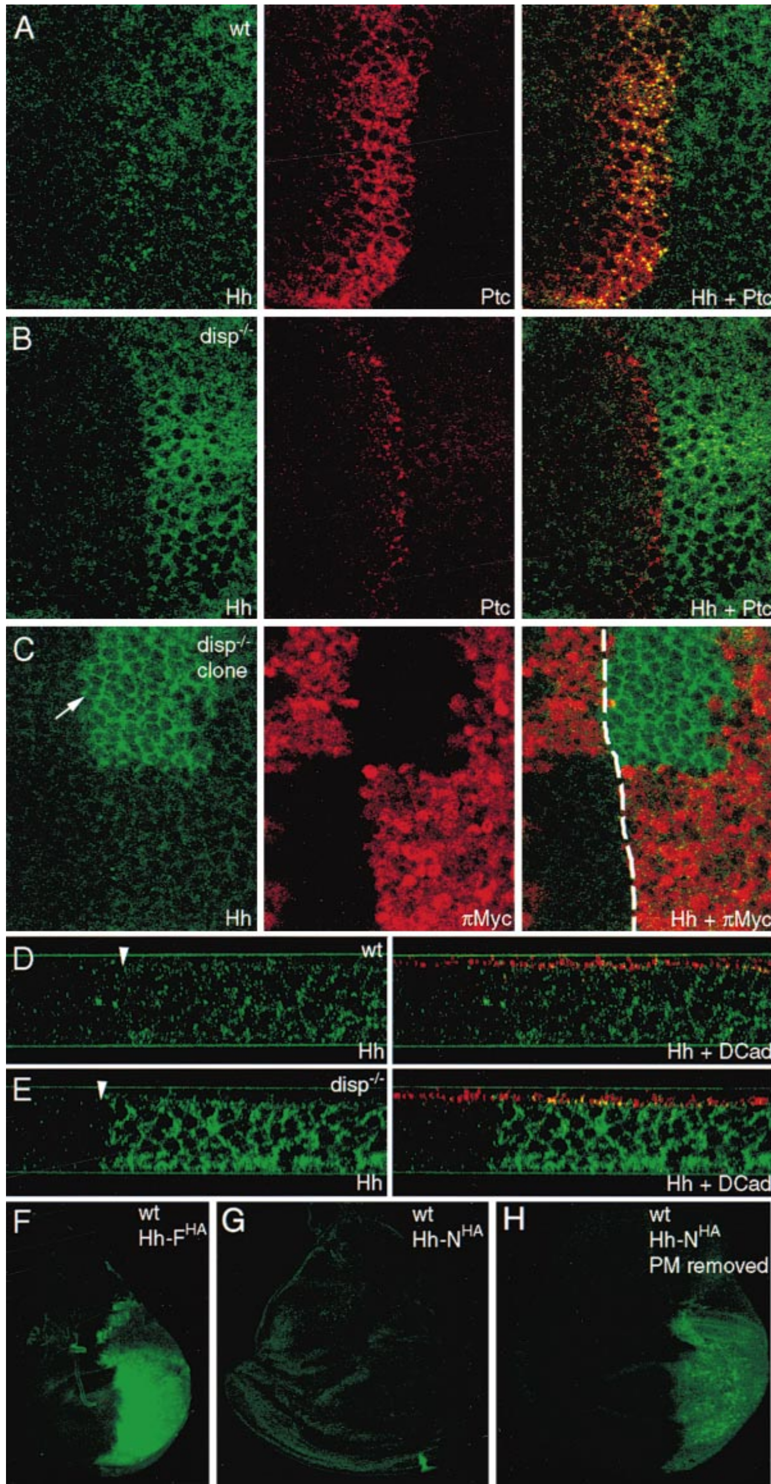

Figure 5. Hh Is Retained in Posterior Cells Mutant for *disp*

(A and B) High magnification view of Hh and Ptc staining in wild-type (A) and *disp* mutant (B) discs. The first panel shows Hh (green), the second Ptc (red), the third both. (A) Note the strong punctate Hh staining colocalizing with high Ptc expression in A compartment cells near the A/P boundary (yellow). (B) Ptc expression in *disp* mutant discs is dramatically reduced. No Hh protein is visible in the A compartment. Hh staining in the P compartment is significantly stronger than in wild-type discs.

(C) $disp^{-/-}$ clones marked by the absence of πMyc (in red). Hh antigen distribution (green) is strongly upregulated in a cell-autonomous manner in posterior *disp* mutant cells (arrow). The A/P compartment boundary is indicated by a dashed line.

(D and E) Optical cross sections of wild-type (D) and *disp* mutant (E) wing discs showing endogenous Hh expression in green (first panel), together with apical DE-Cadherin expression in red (second panel). Arrowheads mark the position of the compartment boundary. In both cases, Hh antigen appears to be distributed over the entire apical/basal axis of P compartment cells, and not localised to a particular region. No Hh is observed in the A compartment of *disp* mutant discs (E), whereas high levels accumulate in cells just anterior to compartment boundary in wild-type discs (D).

(F–H) Wild-type wing imaginal discs expressing *hh-F^HA^* (F) or *hh-N^HA^* (G and H) under *en-Gal4* control. The discs were incubated with α-HA 1° antibody prior to fixation and permeabilization. With the peripodial membrane (PM) intact, Hh-F^{HA} (F) but not Hh-N^{HA} (G) staining was observed on the basal surface. The PM covers the apical disc surface, blocking antibody access. With the PM removed, apical surface Hh-N^{HA} staining could be detected (H). The apical and basal surfaces were defined by their position relative to the peripodial membrane and by cellular mophology. *disp* mutant discs showed the same apical/basal profile of Hh-F^{HA} and Hh-N^{HA} staining, at equivalent levels (not shown).

This lipid modification has been proposed to restrict the range of Hh action, since expression of Hh-Nu results in a spatially extended Hh response in embryos (Porter et al., 1996a). Before assaying the relationship between the cholesterol modification of Hh and the function of Disp, we first further clarified the role of this modification by establishing (1) that in the absence of modification, Hh-Nu possesses a vastly extended range of action in imaginal discs, (2) that this extended range of action is an intrinsic property of Hh-Nu, and (3) that cholesterol-free Hh-Nu is apparently not subject to sequestration by Ptc, yet retains the ability to form vesicular complexes with Ptc in receiving cells.

Expression of Hh-Nu in P cells of the wing imaginal disc results in *dpp-lacZ* expression in the entire A compartment of the disc and a consequent dramatic enlargement of the A compartment (Figure 6C). Thus, Hh-Nu appears to have a range of action at least 5-fold larger

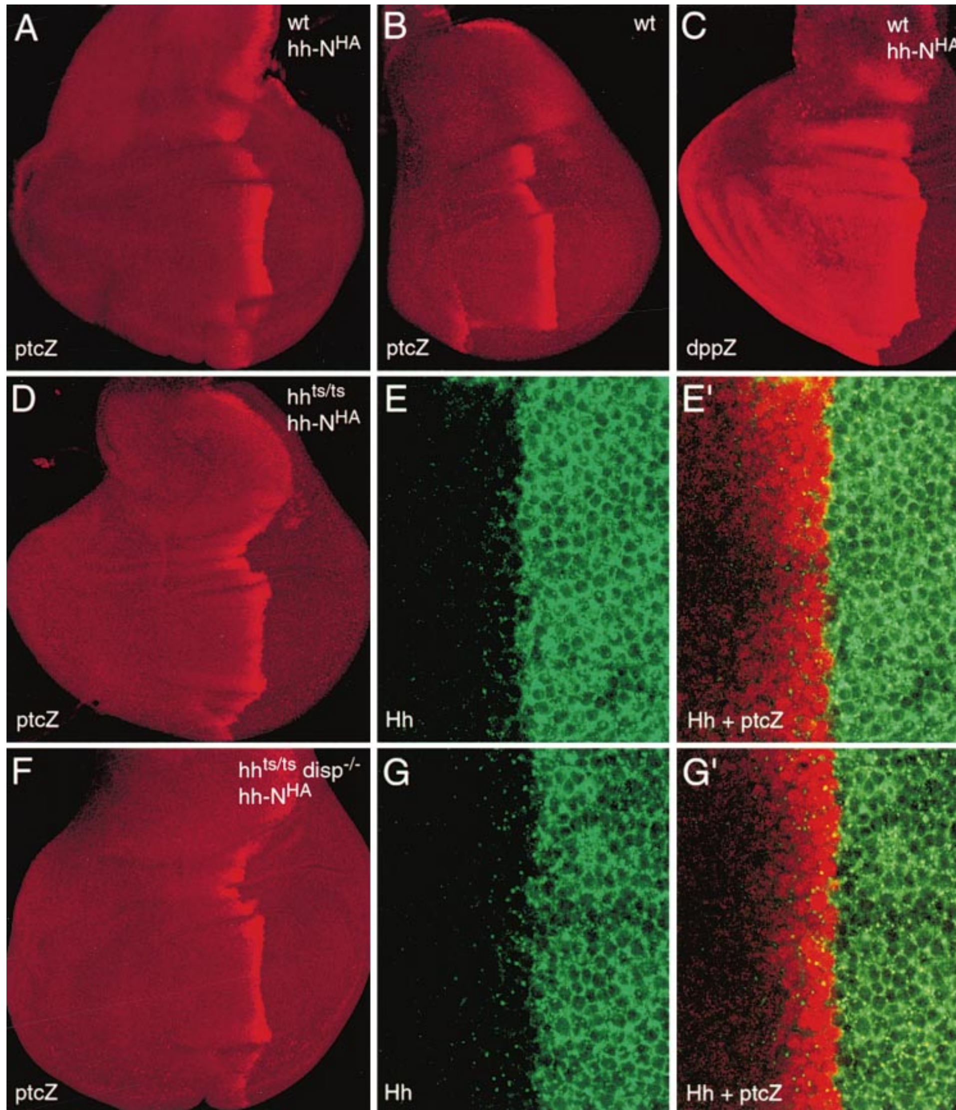

Figure 6. The Cholesterol Anchor Restricts Hh Distribution and Is Responsible for the Retention of Hh in *disp* Mutant Discs

(A and C) Wing imaginal discs expressing *hh-N^{HA}* under *en-Gal4* control. *ptc-lacZ* expression (A) is expanded compared to the wild-type expression pattern (B). *dpp-lacZ* expression (C) fills the entire A compartment.

(D–G) *hh$^{ts2/ts2}$* (D and E) or *hh$^{ts2/ts2}$ disp$^{-/-}$* double (F, G) mutant discs expressing *hh-N^{HA}* under *en-Gal4* control. In the absence of any rescue construct, *hh$^{ts2/ts2}$* larvae have only vestigial imaginal discs when raised at the nonpermissive temperature. *hh-F^{HA}* rescues the growth defect of such discs, albeit with reduced *ptc-lacZ* expression. (D and E) *hh-N^{HA}* also rescues the *hh$^{ts2/ts2}$* phenotype, causing the same anterior outgrowth seen when endogenous Hh is present. *ptc-lacZ* expression is still extended, but not as strongly as in a wild-type disc. (E and E′) High magnification view of (D) shows Hh expression (green) alone and together with *ptc-lacZ* (red, E′). Note the strong punctate Hh staining in A compartment cells, similar to that in wild-type discs (Figure 5). (F and G) The absence of Disp has no apparent effect on Hh-Nu activity. Anterior overgrowth is still observed. (G and G′) High magnification view again shows strong punctate Hh staining in A compartment cells, despite the absence of *disp* activity. (G′) *ptc-lacZ* expression is comparable to that of *hh$^{ts2/ts2}$ disp^{+}* discs (E′).

than that of wild-type Hh. However, since this and previous experiments have been performed in the presence of endogenous Hh, it could not be ruled out that the observed extension of Hh activity depends on, or is even mediated by, endogenous Hh-Np whose range might be expanded in the presence of Hh-Nu. To address this, we created a situation in which Hh-Nu is the sole source of Hh in imaginal discs by expressing *hh-N^{HA}* in P cells under *en-Gal4* in *hh$^{ts2/ts2}$* animals that were shifted to the nonpermissive temperature during early larval stages. Even in the absence of endogenous Hh, Hh-Nu is still capable of inducing the same expanded anterior compartment morphology (Figure 6D) and shows normal punctate staining in anterior cells (Figure 6E). Thus, Hh-Nu alone is able to associate with Ptc and signal in vivo. The cholesterol anchor of Hh appears to be required for the sequestration of Hh by Ptc, since untethered Hh is seemingly unrestricted in its range. We cannot currently rule out the possibility that Hh-Nu is also, at least partially, sequestered by Ptc but that extracellular Hh-Nu levels are abnormally high and saturate the capacity of Ptc. Any sequestration of Hh-Nu must, however, be much less efficient than that of Hh-Np, since even discs containing endogenous Hh plus *en-Gal4* driven Hh-Np do not show the dramatic effect caused by Hh-Nu alone (not shown).

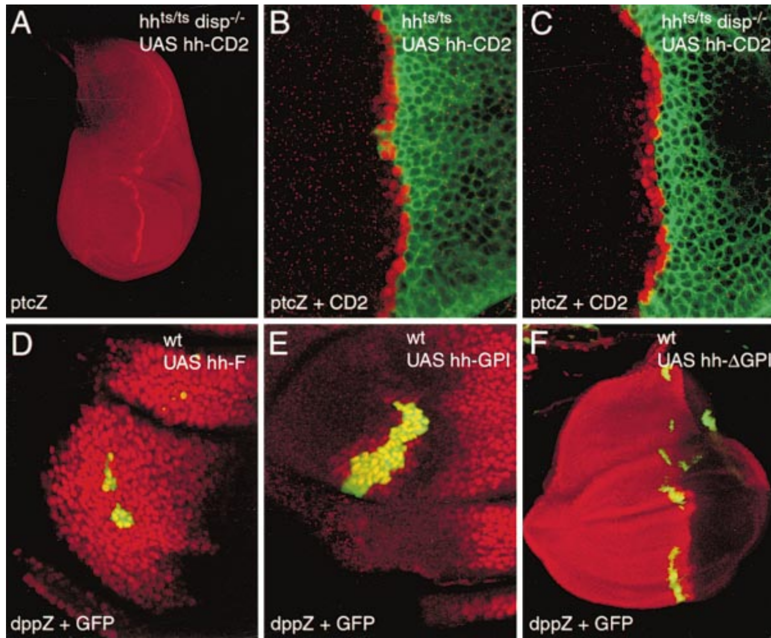

Figure 7. Disp Can Not Release Forms of Hh with Alternative Membrane Anchors

(A–C) *hh*$^{ts2/ts2}$ imaginal discs expressing *UAS-hh-CD2* under *en-Gal4* control. *ptc-lacZ* is shown in red, α-CD2 staining in green. (A) Overview of a *hh*$^{ts2/ts2}$ *disp*$^{-/-}$ disc. The disc is rescued to the same extent as a *hh*$^{ts2/ts2}$ disc. (B and C) High magnification views of a *hh*$^{ts2/ts2}$ (B) and a *hh*$^{ts2/ts2}$ *disp*$^{-/-}$ (C) mutant disc. The activity of Hh-CD2 is unaffected by the absence of *disp*. In both cases (B and C), a single row of *ptc-lacZ* expressing cells at the A/P boundary is observed.

(D–F) Wild-type wing imaginal discs expressing, in marked clones, (D) *UAS-hh-F*, (E) *UAS-hh-GPI*, or (F) *UAS-hh-ΔGPI*. Transgene expression is driven by an *actin5c>CD2>Gal4* transgene (Pignoni and Zipursky, 1997). The clones are marked by the presence of GFP (green), produced from a *UAS-GFP* transgene. *dpp-lacZ* expression is shown in red. Cells expressing both GFP and *dpp-lacZ* appear yellow. (D) Wild-type Hh (*UAS-hh-F*) induces strong *dpp* expression both within the clone and up to 5 or more cell diameters from the expressing cells. (E) Hh-GPI induces a strong *dpp-lacZ* response within the clone and a weaker response in the first row of cells surrounding the clone. (F) Even a small source of untethered Hh-ΔGPI is sufficient to induce *dpp-lacZ* expression in the entire A compartment.

Having confirmed that the cholesterol modification is needed for efficient Hh sequestration but not signaling, we wanted to determine if the retention of Hh in posterior *disp* mutant cells was due to the lipid anchor. We repeated the *hh*$^{ts2/ts2}$ *hh-N*^{HA} experiments in a *disp* mutant background so that the Hh-Nu-secreting P cells were simultaneously lacking endogenous Hh and Disp. The patterning activity of cholesterol-free Hh-Nu was virtually unaffected by the lack of Disp. Hh-Nu caused the same "extended anterior compartment phenotype" in *hh*$^{ts2/ts2}$ *disp*$^{-/-}$ double mutant discs as it caused in the *hh*$^{ts2/ts2}$ single mutant background (Figure 6F). Also, equivalent levels of *ptc-lacZ* expression are induced by Hh-Nu in *hh*$^{ts2/ts2}$ *disp*$^{-/-}$ discs and in *hh*$^{ts2/ts2}$ *disp*$^{+}$ discs, and punctate Hh staining was again observed in anterior cells (Figure 6G). Thus, unlike Hh-Np, cholesterol-free Hh is neither retained nor compromised in its range of action if produced by *disp* mutant cells. Since the sole known structural difference between Hh-Np and Hh-Nu is the C-terminal cholesterol moiety, we conclude that it is this lipid anchor that is responsible for the retention of Hh-Np protein by *disp* mutant cells. From this we infer that the function of Disp is to overcome this retention and thereby permit the release of lipid-modified Hh protein from Hh-producing cells.

Specificity of Disp: GPI-Anchored Hh Is Not Released from the Cell Surface

Having established that the activity of Disp permits the release of tethered Hh protein, we then addressed the specificity of this release mechanism by asking two questions. First, is Hh activity also dependent on Disp if Hh is tethered by a nonlipid anchor? And second, does Disp also liberate Hh protein if Hh is tethered by a lipid anchor other than cholesterol?

To address the first question, we used a fusion protein (Hh-CD2) in which the signaling domain of Hh is fused to the N terminus of the type I transmembrane protein CD2 (Strigini and Cohen, 1997). This derivative of Hh has previously been shown to be effectively tethered to expressing cells and to retain biological activity even in the absence of endogenous Hh (Strigini and Cohen, 1997). We expressed *hh-CD2* under *en-Gal4* control in a *disp*$^{-/-}$ *hh*$^{ts2/ts2}$ mutant background and found that its activity does not depend on the presence of *disp* (Figures 7A and 7C). We therefore conclude that Hh protein with a nonlipid tether—like Hh protein with no tether (Hh-Nu)—functions independently of Disp.

Finally, we asked if addition of lipids other than cholesterol would also tether Hh signaling activity, and whether such tethering can be overcome by Disp. We generated a form of Hh-N (Hh-GPI) that carries the C-terminal 54 residues of *Drosophila* Fasciclin I (Fas1, Zinn et al., 1988), including the glycosyl-phosphatidylinositol (GPI)-anchoring signal of Fas1. As a control we used a derivative of Hh-GPI (Hh-ΔGPI) in which the GPI-anchoring signal was mutated. When Hh-ΔGPI is expressed in marked clones of wing imaginal disc cells, ubiquitous expression of *dpp-lacZ* is observed in the entire A compartment, which is extended in size (Figure 7F). This phenotype is the same as that of Hh-Nu and indicates that the addition of heterologous sequences does not compromise the long-range signaling activity of Hh-Nu. In sharp contrast, expression of Hh-GPI induces ectopic *dpp-lacZ* expression only in Hh-GPI-expressing cells and in their immediate wild-type neighbors (Figure 7E). Conversely, wild-type Hh in the same assay induces *dpp-lacZ* in wild-type cells up to five or more cell diameters away (Figure 7D). Thus, the GPI moiety effectively tethers Hh to the surface of expressing cells. Disp, which

is present and active in these cells, can not liberate Hh-GPI as it does Hh-Np, indicating that cholesterol is an important determinant of the Disp-dependent release mechanism of tethered Hh.

Discussion

Here we describe the identification of a novel segment-polarity gene, *disp*, and show that it functions in the Hh signaling pathway. The analysis of genetic mosaics indicates that *disp* acts exclusively in Hh-secreting cells and is dispensable in Hh-receiving cells, placing its action upstream of the Hh coreceptors Ptc and Smo. The observation that lack of *disp* function does not affect *hh* transcription further narrows the window in which Disp may play a role to the processes of Hh biogenesis and release. Biochemical analysis, however, rules out a function for Disp in the generation of Hh-Np by proteolytic processing, and no differences could be observed in the subcellular localization of Hh between secreting wild-type and *disp* mutant cells. Two key findings of our analysis eventually pinpoint the role of Disp: the observation that *disp* mutant cells retain rather than secrete Hh; and the demonstration that Hh-Nu, the cholesterol-free variant of Hh-Np, bypasses the requirement for Disp. From these two results, we conclude that the normal function of Disp is to liberate the cholesterol-tethered form of Hh from internal or surface membranes of producing cells.

Specificity of Disp Activity: Dedication to Hh and Cholesterol

One surprising observation made in the course of our analysis of Disp concerns the specificity of its action. First, it appears to be required exclusively for the release of Hh protein, and not for that of other signaling proteins, or for any essential physiological process. This conclusion is drawn from the observation that animals in which the wild-type *disp* gene is replaced by a transgene operating only in P cells develop and function normally, despite the absence of *disp* product in all A cells. An additional level of specificity can be observed in the target of Disp activity. Disp can liberate Hh protein tethered by cholesterol, but not that anchored by glycosyl-phosphatidylinositol. We do not know whether it is the presence of an SSD in Disp that governs its selectivity with respect to lipid anchors. It is interesting to note that the processing or release of other important signaling molecules also depends on dedicated multitransmembrane domain proteins in the secreting cells: the Wingless signal requires the activity of Porcupine (van den Heuvel et al., 1993; Kadowaki et al., 1996), and the EGF signal Spitz only becomes an effective ligand in the presence of Rhomboid (Golembo et al., 1996; Wasserman and Freeman, 1998). It is not known yet to what extent Porcupine and Rhomboid affect processing versus release of their respective ligands.

The Dilemma of Restricting Versus Liberating Hh

Hh is an extremely powerful signaling molecule. When unrestricted, it is able to move to and program cells far away from its origin of synthesis. Two mechanisms have evolved to restrict its range of action: (1) upregulation of *ptc* transcription with subsequent sequestration of Hh ligand by Ptc protein and (2) the employment of a lipid tether. Intriguingly, it appears that to function efficiently, the two mechanisms depend on each other. For example, if P cells secrete Hh protein that lacks the cholesterol tether, this Hh protein reaches the entire population of A compartment cells despite the presence of Ptc protein at the boundary (Figure 6C). Conversely, in the absence of Ptc, the cholesterol moiety does not function as an anchor, since under such conditions even wild-type Hh protein can reach over impressively large distances (Figure 3D, Chen and Struhl, 1996). Why are these two mechanisms interdependent? The answer is not known, but it is intriguing that both the lipid tether of Hh as well as the SSD of Ptc point to the involvement of cholesterol as a common link.

Lipid modifications are employed to tether proteins to cellular membranes. In many instances these modifications fulfill the function of a posttranslationally added transmembrane domain. The use of a cholesterol tether for a signaling molecule that has to act at a distance is unprecedented. Since its discovery, it has posed a paradox: how can Hh be released from cells if it is covalently attached to a cholesterol moiety that is presumably inserted in lipid bilayers? Several different scenarios can be envisaged. Either Hh-N is liberated from its anchor by a cleavage event involving C-terminal proteolysis or anchor hydrolysis, or Hh-Np is released by displacing the cholesterol anchor from the lipid membrane or by the formation of extracellular microparticles through membrane vesiculation. Biochemical analyses in cultured cells and the properties of Hh in vivo argue against a cleavage event. The vast majority of Hh protein expressed in cultured cells is cell associated and not soluble (Pepinsky et al., 1998). Moreover, two forms of Hh that carry a membrane anchor other than cholesterol—Hh-CD2 with a transmembrane domain and Hh-GPI with a glycosyl-phosphatidylinositol moiety—are not released from the cell surface, ruling out proteolysis as the mechanism of Hh release. We note that the possibility can not be excluded that a small fraction of total Hh is indeed secreted without cholesterol tether and that only this fraction of Hh is active. However, in our hands, even high amounts of such a form (Hh-Nu) do not induce wild-type levels of target gene expression, despite the extended range of action (see Figure 6). Thus, we favor the view that the active form that is released from Hh-secreting cells is Hh-Np and that the mechanism of release involves a displacement of its cholesterol tether or some form of membrane vesiculation.

SSD Proteins and Hh Signaling

Here we show that the mechanism of Hh release does not operate effectively in the absence of the multi-transmembrane domain protein Disp. Our results reveal the existence of a specific pathway by which Hh overcomes its cholesterol impediment. Unexpectedly, the molecular analysis of the *disp* gene indicates that this pathway of Hh release—like that of Hh restriction—involves an SSD protein. However, although Ptc and Disp are structurally similar, the two proteins play opposite roles. Ptc is required in A cells to *sequester* Hh-Np,

and Disp is required in P cells to *displace* Hh-Np. In spite of these seemingly inverse roles, an intriguing parallel exists between Ptc and Disp in that both SSD proteins appear to depend on the cholesterol modification of Hh to exert their function: Ptc efficiently sequesters cholesterol-modified Hh but has little effect on Hh-Nu, and Disp enables the efficient release of cholesterol-modified Hh but has no apparent effect on the release of Hh-Nu. It is not known whether the SSDs of Ptc and Disp play a direct role in binding cholesterol-modified Hh. If this were the case, one could envisage an equilibrium between membrane cholesterol (chol), lipid bilayer-associated Hh-Np, Disp-associated Hh-Np, and free Hh-Np, in which Disp functions as a catalyst for Hh release:

$$\text{bilayer} \cdot \text{Hh-Np} + \text{Disp} \cdot \text{chol} \rightleftharpoons \text{bilayer} \cdot \text{chol} + \text{Disp} \cdot \text{Hh-Np} \rightleftharpoons \text{bilayer} + \text{Disp} \cdot \text{chol} + \text{free Hh-Np}$$

As the affinity of Hh-Np would have to be higher to Disp than to the lipid bilayer, the presence of a cofactor for the dissociation of Hh-Np from Disp must also be postulated. Once Hh-Np is released, reinsertion of it into membranes of nearby A cells might be hindered by an association with carrier proteins or proteoglycans, whose synthesis might depend on the function of Tout-velu, an EXD protein required for proper movement of Hh (Bellaiche et al., 1998). In a reverse sequence of events, Ptc could be involved in reinserting Hh-Np into the lipid bilayer of receiving cells.

An alternative, yet not mutually exclusive, possibility is that the SSD of Disp plays an important regulatory function. As has also been proposed for Ptc (Beachy et al., 1997), the SSD of Disp could be involved in monitoring membrane sterol levels, thereby assessing the metabolic state of cells before allowing the release of the Hh signal. Whatever the precise molecular mechanism by which cells release cholesterol-tethered Hh, it becomes apparent that the unusual way of restricting the range of a signal by means of a lipid anchor has its price. It requires at least one dedicated component, Disp.

Experimental Procedures

Drosophila Stocks and Clonal Analysis

In a large collaborative effort involving all authors, 1737 lethal P element insertions created by Deák and coworkers (Deák et al., 1997) were recombined onto FRT80 or FRT82 chromosomes to allow the generation of somatic and germline clones by Flp-mediated mitotic recombination (Xu and Rubin, 1993; Chou and Perrimon, 1996). Recombinants were generated using the *eyFLP* system (Newsome et al., 2000) to efficiently identify by their mosaic eye color those animals with both the FRT and *P[w⁺]* insertions on the same arm. The P element *l(3)S037707* was then identified by screening these lines for *hh*-like phenotypes. Below, we list the genotypes used in our analysis:

- *disp* germline clones:
  y w hsp70-flp; FRT82 *disp*/FRT82 *P[ovo^D]* × FRT82 *disp*/TM3 *P[y$^+$]*
- *disp* clones in adult wings:
  f hsp70-flp; FRT82 *disp*/FRT82 *M(3R)w124 P[f$^+$]*
- *hh* clones in adult wings:
  y w hsp70-flp; FRT82 *hh^{AC}*/FRT82 *M(3R)w124 P[y$^+$]*
- *disp* clones in imaginal discs, using *dpp-lacZ* or *ptc-lacZ*:
  y w hsp70-flp; *dpp(ptc)-lacZ*; FRT82 *disp*/FRT82 *M(3R)w124 2x P[hsπMyc, w$^+$]*
- *disp* clones in imaginal discs, *hh-lacZ*:
  y w hsp70-flp; FRT82 *disp hh-lacZ*/FRT82 *P[hsCD2, y$^+$] hh-lacZ*
- *hh^{ts2}* rescue experiments:
  en-Gal4/*UAS-hh-F(N^{HA})*; *hh^{ts2}*/*hh^{ts2}*
  en-Gal4/*UAS-hh-F(N^{HA})*; *disp hh^{ts2}*/*disp hh^{ts2}*
  en-Gal4/*UAS-hh-CD2*; *hh^{ts2}*/*hh^{ts2}*
  en-Gal4/*UAS-hh-CD2*; *disp hh^{ts2}*/*disp hh^{ts2}*
- clones expressing Hh, Hh-GPI, or Hh-ΔGPI in imaginal discs:
  y w hsp70-flp; *dpp-lacZ*/*UAS-hh (hh-GPI, hh-ΔGPI)*; *actin5c>CD2>Gal4 UAS-GFP*

hh-temperature-sensitive animals were generated using the *hh^{ts2}* allele (Ma et al., 1993) balanced over the TM6b[*Tb*] balancer. These animals were allowed to develop at the permissive temperature (18°C) for 2 to 4 days before shifting to the nonpermissive temperature (29°C). Imaginal discs were dissected and fixed after 3 to 4 days at 29°C.

Transgenes

Reporter genes used in this study were *dpp-lacZ^{P10638}* (Zecca et al., 1995), *hh^{P30}* (Lee et al., 1992), and *ptc(10.8L)A* (Chen and Struhl, 1996). The *UAS-hh* transgenes used were derived from the full-length *hh* cDNA clone 11 (Lee et al., 1992). *UAS-hh-F^{HA}* contains the full-length cDNA, whereas *UAS-hh-N^{HA}* is truncated and contains a stop codon following residue G257, the normal site of Hh autoproteolytic cleavage (Porter et al., 1995). Both transgenes contain a triple HA–epitope tag inserted between *hh* codons 254 and 255. The *UAS-hh-GPI* transgene contains sequences of the *fasciclin I* gene (Zinn et al., 1988) encoding the C-terminal-most 54 amino acid (aa) residues fused to Hh at G257. The *UAS-hh-ΔGPI* harbors the same fusion, but the last 27 residues of Fasciclin I are replaced by a stop codon. The *UAS-hh-CD2* transgene was a gift from M. Strigini (Strigini and Cohen, 1997).

The *tub-disp* rescue construct contains the full-length *disp* cDNA flanked at its 3′ end by the 3′ UTR of the *tubulinα1* gene. In the *UAS-disp* transgene, a triple HA tag was inserted in frame at the 3′ end of the open reading frame, followed by the 3′ *tubulinα1* UTR.

All constructs were inserted into pUAST (Brand and Perrimon, 1993) or into a P element plasmid containing the promoter of the *tubulinα1* gene (Basler and Struhl, 1994).

Molecular Cloning

Genomic DNA from either side of the P element *l(3)S03770* was obtained by plasmid rescue upon restriction by BamHI or EcoRI. Sequence analysis of rescued fragments revealed that the P element was inserted within sequences of a *Drosophila Yoyo* transposable element, which in turn were flanked on either side by sequences identical to an EST in the Berkeley Drosophila Genome Databank (clone No. ld12634). Thus, the P element *l(3)S03770* is inserted within an intron of the *disp* gene, located at position aa 812. A 0.8 kb EcoR1 to Spe1 fragment of this EST clone was used to probe an embryonic 0–8 hr cDNA library. Ten positive clones were picked and sequenced. From these new clones and the original EST clone, a composite full-length sequence was assembled with an ORF predicting a protein of 1218 aa in length. BLAST analysis of the sequence revealed low homology (smallest sum probability (SSP) score of $\sim 1 \times 10^{-8}$) to Mouse NPC1 and lower similarity to Human NPC1(SSP $\sim 1 \times 10^{-6}$) and *Drosophila* Ptc (SSP $\sim 1 \times 10^{-5}$). The highest homology was to a predicted but as yet uncharacterized *C. elegans* protein (GenBank acc. no. AAC48001, SSP $\sim 1 \times 10^{-55}$). Three independent transmembrane (TM) domain prediction programs (TopPred 2, TMHMM, HMMTOP) all predicted 12 TM domains in the Disp protein. Mobilization of the original P element insertion resulted in several independent deletions, removing sequences C-terminal to the P insertion at position aa 812. All these deletions resulted in the same pupal lethality and small disc phenotype of the original P element–induced mutation.

Immunoblotting and Histochemistry

Protein was prepared from dissected third instar larvae by boiling for 5 minutes in 1× SDS sample buffer (20 larvae/100 μl). Protein samples were run on a 17% acrylamide gel (20 μl wild type; 30 μl *disp*$^{-/-}$), then transferred to nylon membranes. Membranes were blocked, then incubated with mouse α-HA 11 antibody (Babco, 1:1000) followed by α-HRP 2° antibody (1:10000). Immunoreactive proteins were visualized by chemiluminescence (ECL, Amersham).

Imaginal discs from third instar larvae were fixed and stained by standard techniques, except when using the rabbit α-Hh protein, in which case discs were fixed for 20 min in 4% PFA in PBS. Cell surface staining was assayed as follows: imaginal discs were incubated 30 min at room temperature in α-HA (1:1000) followed immediately by fixation for 30 min in 2% formaldehyde in PBS. Subsequent procedures were the same as with standard preparations. Antibodies were mouse monoclonal α-Ptc (gift from I. Guerrero), α-CD2 OX34 (Serotec), α-HA 12C5 (Boehringer/Roche), α-Myc 9E10, and α-βGal (Promega); rabbit polyclonal α-Hh (gift from P. Ingham and P. Thérond) and α-βGal (Cappel); rat monoclonal α-HA (Boehringer/Roche) and α-DE-Cadherin (gift from H. Oda); and Alexa 488 and 594 fluorescent 2° antibodies (Molecular Probes).

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References

Basler, K., and Struhl, G. (1994). Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein. Nature *368*, 208–214.

Beachy, P.A., Cooper, M.K., Young, K.E., von Kessler, D.P., Park, W.J., Hall, T.M., Leahy, D.J., and Porter, J.A. (1997). Multiple roles of cholesterol in hedgehog protein biogenesis and signaling. Cold Spring Harb. Symp. Quant. Biol. *62*, 191–204.

Bellaiche, Y., The, I., and Perrimon, N. (1998). Tout-velu is a Drosophila homologue of the putative tumour suppressor EXT-1 and is needed for Hh diffusion. Nature *394*, 85–88.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Capdevila, J., and Guerrero, I. (1994). Targeted expression of the signaling molecule decapentaplegic induces pattern duplications and growth alterations in Drosophila wings. EMBO J. *13*, 4459–4468.

Carstea, E.D., et al. (1997). Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis. Science *277*, 228–231.

Chen, Y., and Struhl, G. (1996). Dual roles for patched in sequestering and transducing hedgehog. Cell *87*, 553–563.

Chen, Y., and Struhl, G. (1998). In vivo evidence that patched and smoothened constitute distinct binding and transducing components of a hedgehog receptor complex. Development *125*, 4943–4948.

Chou, T.B., and Perrimon, N. (1996). The autosomal FLP-DFS technique for generating germline mosaics in Drosophila melanogaster. Genetics *144*, 1673–1679.

Couso, J.P., Bishop, S.A., and Martinez Arias, A. (1994). The wingless signaling pathway and the patterning of the wing margin in Drosophila. Development *120*, 621–636.

Deák, P., et al. (1997). P-element insertion alleles of essential genes on the third chromosome of Drosophila melanogaster: correlation of physical and cytogenetic maps in chromosomal region 86E–87F. Genetics *147*, 1697–1722.

Forbes, A.J., Nakano, Y., Taylor, A.M., and Ingham, P.W. (1993). Genetic analysis of hedgehog signaling in the Drosophila embryo. Development (Suppl.), 115–124.

Gil, G., Faust, J.R., Chin, D.J., Goldstein, J.L., and Brown, M.S. (1985). Membrane-bound domain of HMG CoA reductase is required for sterol-enhanced degradation of the enzyme. Cell *41*, 249–258.

Golembo, M., Raz, E., and Shilo, B.-Z. (1996). The Drosophila embryonic midline is the site of Spitz processing, and induces activation of the EGF receptor in the ventral ectoderm. Development *122*, 3363–3370.

Goodrich, L.V., Johnson, R.L., Milenkovic, L., McMahon, J.A., and Scott, M.P. (1996). Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by hedgehog. Genes Dev. *10*, 301–312.

Hahn, H., Christiansen, J., Wicking, C., Zaphiropoulos, P.G., Chidambaram, A., Gerrard, B., Vorechovsky, I., Bale, A.E., Toftgard, R., Dean, M., et al. (1996). A mammalian patched homolog is expressed in target tissues of sonic hedgehog and maps to a region associated with developmental abnormalities. J. Biol. Chem. *271*, 12125–12128.

Hammerschmidt, M., Brook, A., and McMahon, A.P. (1997). The world according to hedgehog. Trends Genet. *13*, 14–21.

Hooper, J.E., and Scott, M.P. (1989). The Drosophila patched gene encodes a putative membrane protein required for segmental patterning. Cell *59*, 751–765.

Hua, X., Nohturfft, A., Goldstein, J.L., and Brown, M.S. (1996). Sterol resistance in CHO cells traced to point mutation in SREBP cleavage-activating protein. Cell *87*, 415–426.

Ingham, P.W. (1998). Transducing Hedgehog: the story so far. EMBO J. *17*, 3505–3511.

Kadowaki, T., Wilder, E., Klingensmith, J., Zachary, K., and Perrimon, N. (1996). The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in wingless processing. Genes Dev. *10*, 3116–3128.

Lawrence, P.A., and Struhl, G. (1996). Morphogens, compartments, and pattern: lessons from drosophila? Cell *85*, 951–961.

Lecuit, T., Brook, W.J., Ng, M., Calleja, M., Sun, H., and Cohen, S.M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the Drosophila wing. Nature *381*, 387–393.

Lee, J.J., von Kessler, D.P., Parks, S., and Beachy, P.A. (1992). Secretion and localized transcription suggest a role in positional signaling for products of the segmentation gene hedgehog. Cell *71*, 33–50.

Lee, J.J., Ekker, S.C., von Kessler, D.P., Porter, J.A., Sun, B.I., and Beachy, P.A. (1994). Autoproteolysis in hedgehog protein biogenesis. Science *266*, 1528–1537.

Liscum, L., and Klansek, J.J. (1998). Niemann-Pick disease type C. Curr. Opin. Lipidol. *9*, 131–135.

Loftus, S.K., Morris, J.A., Carstea, E.D., Gu, J.Z., Cummings, C., Brown, A., Ellison, J., Ohno, K., Rosenfeld, M.A., Tagle, D.A., et al. (1997). Murine model of Niemann-Pick C disease: mutation in a cholesterol homeostasis gene. Science *277*, 232–235.

Ma, C., Zhou, Y., Beachy, P.A., and Moses, K. (1993). The segment polarity gene hedgehyg is required for progression of the morphogenetic furrow in the developing Drosophila eye. Cell *75*, 927–938.

Marigo, V., Scott, M.P., Johnson, R.L., Goodrich, L.V., and Tabin, C.J. (1996). Conservation in hedgehog signaling: induction of a chicken patched homolog by Sonic hedgehog in the developing limb. Development *122*, 1225–1233.

Méthot, N., and Basler, K. (1999). Hedgehog controls limb development by regulating the activities of distinct transcriptional activator and repressor forms of Cubitus interruptus. Cell *96*, 819–831.

Mullor, J.L., Calleja, M., Capdevila, J., and Guerrero, I. (1997). Hedgehog activity, independent of decapentaplegic, participates in wing disc patterning. Development *124*, 1227–1237.

Murone, M., Rosenthal, A., and de Sauvage, F.J. (1999). Sonic hedgehog signaling by the patched-smoothened receptor complex. Curr. Biol. *9*, 76–84.

Nakano, Y., Guerrero, I., Hidalgo, A., Taylor, A., Whittle, J.R., and Ingham, P.W. (1989). A protein with several possible membrane-spanning domains encoded by the Drosophila segment polarity gene patched. Nature *341*, 508–513.

Nellen, D., Burke, R., Struhl, G., and Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. Cell *85*, 357–368.

Neumann, C., and Cohen, S. (1997). Morphogens and pattern formation. Bioessays *19*, 721–729.

Newsome, T.P., Åsling, B., and Dickson, B.J. (2000). Analysis of Drosophila photoreceptor axon guidance in eye-specific mosaics. Development, in press.

Nüsslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. Nature *287*, 795–801.

Osborne, T.F., and Rosenfeld, J.M. (1998). Related membrane domains in proteins of sterol sensing and cell signaling provide a glimpse of treasures still buried within the dynamic realm of intracellular metabolic regulation. Curr. Opin. Lipidol. *9*, 137–140.

Pepinsky, R.B., Zeng, C., Wen, D., Rayhorn, P., Baker, D.P., Williams, K.P., Bixler, S.A., Ambrose, C.M., Garber, E.A., Miatkowski, K., et al. (1998). Identification of a palmitic acid-modified form of human sonic hedgehog. J. Biol. Chem. *273*, 14037–14045.

Pignoni, F., and Zipursky, S.L. (1997). Induction of Drosophila eye development by decapentaplegic. Development *124*, 271–278.

Porter, J.A., von Kessler, D.P., Ekker, S.C., Young, K.E., Lee, J.J., Moses, K., and Beachy, P.A. (1995). The product of hedgehog autoproteolytic cleavage active in local and long-range signaling. Nature *374*, 363–366.

Porter, J.A., Ekker, S.C., Park, W.J., von Kessler, D.P., Young, K.E., Chen, C.H., Ma, Y., Woods, A.S., Cotter, R.J., Koonin, E.V., et al. (1996a). Hedgehog patterning activity: role of a lipophilic modification mediated by the carboxy-terminal autoprocessing domain. Cell *86*, 21–34.

Porter, J.A., Young, K.E., and Beachy, P.A. (1996b). Cholesterol modification of hedgehog signaling proteins in animal development. Science *274*, 255–259.

Sanchez-Herrero, E., Couso, J.P., Capdevila, J., and Guerrero, I. (1996). The fu gene discriminates between pathways to control dpp expression in Drosophila imaginal discs. Mech. Dev. *55*, 159–170.

Slusarski, D.C., Motzny, C.K., and Holmgren, R. (1995). Mutations that alter the timing and pattern of cubitus interruptus gene expression in Drosophila melanogaster. Genetics *139*, 229–240.

Strigini, M., and Cohen, S.M. (1997). A hedgehog activity gradient contributes to AP axial patterning of the Drosophila wing. Development *124*, 4697–4705.

Tabata, T., and Kornberg, T.B. (1994). Hedgehog is a signaling protein with a key role in patterning Drosophila imaginal discs. Cell *76*, 89–102.

Taylor, A.M., Nakano, Y., Mohler, J., and Ingham, P.W. (1993). Contrasting distributions of patched and hedgehog proteins in the Drosophila embryo. Mech. Dev. *42*, 89–96.

van den Heuvel, M., Harryman-Samos, C., Klingensmith, J., Perrimon, N., and Nusse, R. (1993). Mutations in the segment polarity genes wingless and porcupine impair secretion of the wingless protein. EMBO J. *12*, 5293–5302.

von Heijne, G. (1992). Membrane protein structure prediction, hydrophobicity analysis and the positive-inside rule. J. Mol. Biol. *225*, 487–494.

Wasserman, J.D., and Freeman, M. (1998). An autoregulatory cascade of EGF receptor signaling patterns the Drosophila egg. Cell *95*, 355–364.

Xu, T., and Rubin, G.M. (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. Development *117*, 1223–1237.

Zecca, M., Basler, K., and Struhl, G. (1995). Sequential organizing activities of engrailed, hedgehog and decapentaplegic in the Drosophila wing. Development *121*, 2265–2278.

Zinn, K., McAllister, L., and Goodman, C.S. (1988). Sequence analysis and neuronal expression of fasciclin I in grasshopper and Drosophila. Cell *53*, 577–587.

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