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Requirements for hedgehog movement and signaling in Drosophila

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Requirements for Hedgehog Movement and Signaling in *Drosophila*

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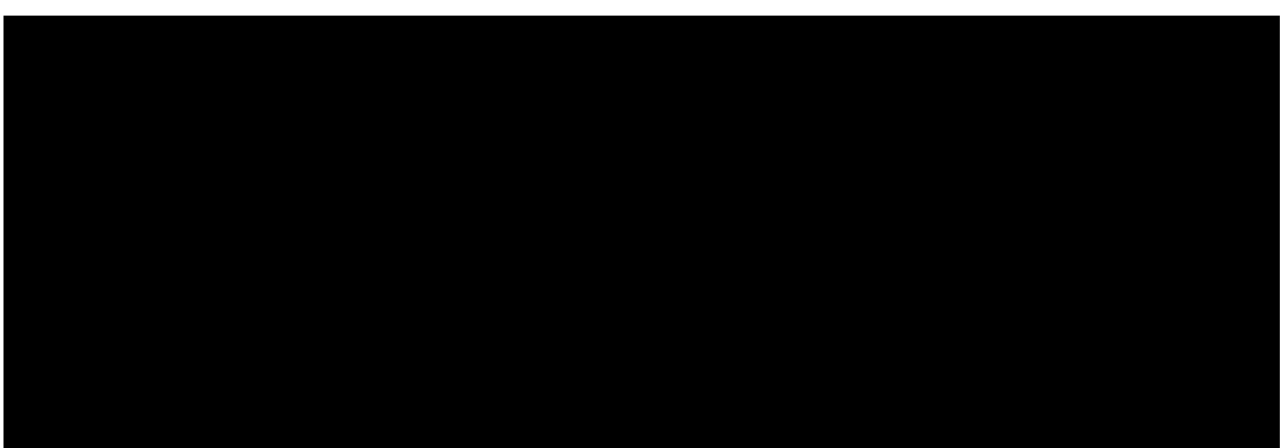

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Abstract

Hedgehog (Hh) is a secreted protein that is required for many developmental processes, in vertebrates as well as in *Drosophila*. Despite its importance, the mechanisms for the reception of Hh signal by cells not immediately adjacent to its source are poorly understood. The mature Hh protein contains two hydrophobic modifications: a palmitic acid moiety which is added to the N-terminus, and a cholesterol moiety at the C-terminus. These modifications give Hh a high affinity for cell membranes, raising the question of how it is able to travel far enough away from the cells that produce it to signal throughout its known range.

I used a cell culture based assay to approach this problem, comparing the activity of wild-type Hh (HhNp) to a truncated version which is not modified by cholesterol (HhN). Although both molecules are capable of signaling in an autocrine fashion in transfected cells that are producing Hh, only HhN is capable of paracrine signaling when naive cells are treated with conditioned media. I attempted to develop assay conditions under which signaling by HhNp can occur, and found that cells producing HhNp can not signal to naive cells across a membrane. However, when the two types of cells are cultured together, signaling can occur, suggesting that cell-cell contact is important for paracrine signaling by HhNp.

In addition to these experiments, I examined the role of endocytosis in Hh movement and signaling. Using a temperature sensitive form of dynamin, I selectively inactivated endocytosis in Hh receiving cells *in vivo*. My data did not support a role for endocytosis in Hh signaling, or the movement of Hh away from producing cells, instead providing evidence that it is required for proper sequestration of the protein.

Lastly, I looked at the function of the hydrophobic modifications of Hh. I examined the effects of loss of one, or both, modifications on Hh distribution and signaling potency in cultured cells and in flies. Together, my results argue that Hh movement is a complex process, and argue against diffusion as an important mechanism for Hh distribution.

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Chapter 1

Introduction

Developmental biology is the study of how one cell grows into a multicellular organism. This process encompasses many separate aspects, including specification of the anterior-posterior and dorsal-ventral axes, regulation of cell growth and proliferation, pattern formation, cell fate determination, and differentiation. Of particular interest is the problem of pattern formation, or how each cell in a developmental field knows where it is in relation to other cells, and therefore which of many possible fates to adopt. This problem has been studied from the very earliest days of experimental developmental biology.

Early embryologists argued over whether development is mosaic, meaning that individual regions of the embryo develop independently from each other, or regulative, with specification of cell type occurring through cell-cell interactions. Experiments to resolve this question involved disrupting early embryos to observe whether individual regions could develop separately, but these gave conflicting results depending on the organism used and the method of disruption. The work of Spemann and Mangold, published in 1924, introduced the concept of the embryonic organizer, a group of cells that has the capacity to induce other cells to adopt a particular fate. Examples of organizers include the dorsal blastopore lip (also called Spemann's organizer), which induces gastrulation, the zone of polarizing activity (ZPA) and apical epidermal ridge (AER), which together guide pattern formation and outgrowth in the vertebrate limb, and the compartment borders of fly imaginal discs. Particularly intriguing is the fact that induction by an organizer leads to the production of many diverse cell types, which arise from different areas of the embryo. While the

importance of organizers in development was soon established, the nature and mechanism of this organizing activity remained unknown.

One early model for the spread of positional information, and the development of different cell fates, was the concept of a morphogen (reviewed in Neumann and Cohen 1997). Morphogens were proposed to be secreted molecules that diffuse out from a source and produce a concentration gradient that can be interpreted by cells in a concentration dependant manner. Recently, biochemical and genetic studies of development have identified many secreted molecules that contribute to the inducing activities of organizers. These include TGF-β family members (particularly Dpp and the BMP proteins), Wingless, FGF, Hedgehog and many others. While the requirement for these molecules in development has been well established, the exact mechanism by which they carry positional information to cells far from their source remains unclear.

One molecule for which gradient signaling has been established is Bicoid, a protein required for establishment of the anterior-posterior axis in the fly oocyte (reviewed in (Ephrussi and St Johnston 2004). Messenger RNA for bicoid is produced by maternal cells during oocyte development, and deposited into the anterior end of the oocyte. After fertilization, Bicoid protein diffuses through the embryo, forming a concentration gradient (Driever and Nusslein-Volhard 1988). The protein acts as a transcription factor and binds directly to DNA to regulate gene expression; high concentrations of Bicoid promote expression of anterior genes, while lower concentrations lead to expression of more posterior genes (Struhl, Struhl et al. 1989). In combination with posterior group genes such as *oskar*, the Bicoid gradient leads to establishment of the anterior-posterior axis along the length of the embryo. This is a classic example of morphogen signaling. However, Bicoid distribution

functions in the syncytial blastoderm stage, before the onset of cellularization. This means that there are few obstacles, such as cell membranes or extracellular matrix, to impede the diffusion of Bicoid protein. It is important to note that this mechanism may not be applicable to the distribution of other morphogens that function later in development.

In recent years there have been attempts to determine how other putative morphogens localize *in vivo*, using either antibodies or GFP fusion proteins. For instance, when a Dpp-GFP was expressed in Drosophila a graded distribution pattern was observed (Entchev, Schwabedissen et al. 2000). Other proteins, such as activin, have been found to have concentration-dependent effects in cultured explant systems (Dyson and Gurdon 1998). Still, little remains known about how such gradients might arise in living tissue.

Hedgehog in Development

My work has focused on the study of the movement and activity of one of these putative morphogens, Hedgehog. Hedgehog (Hh) is a conserved protein critically important for development in many organisms from flies to vertebrates. In vertebrates, Hh proteins are involved in multiple processes including establishment of dorsal-ventral polarity in the neural tube (Roelink, Porter et al. 1995), limb development (Riddle, Johnson et al. 1993), and bone development (Vortkamp, Lee et al. 1996). Additionally, defects in the Hh signaling pathway have been linked to various cancers, such as medulloblastomas (Goodrich, Milenkovic et al. 1997) and basal cell nevus syndrome (Oro, Higgins et al. 1997). In *Drosophila*, Hh is required for proper segmentation in the embryo (Nusslein-Volhard and Wieschaus 1980), as well as anterior-posterior patterning in the imaginal discs (Tabata and Kornberg 1994), the larval tissues that will contribute to the adult animal.

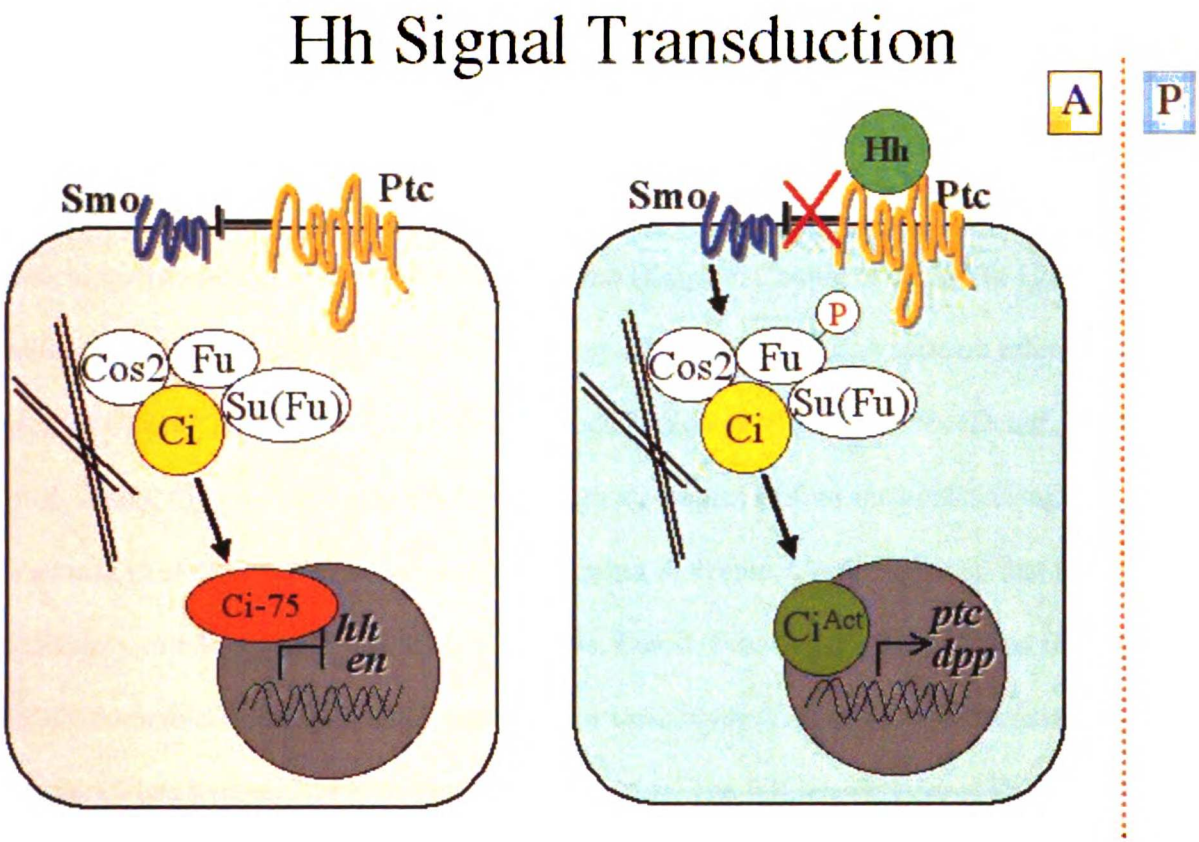


Figure 1. The Hh signal transduction pathway in the anterior compartment of the *Drosophila* imaginal disc
Courtesy of Ansgar Klebes and Brenda Ng

Reception of the Hh signal is a complex process, and even after years of research, many gaps remain in our understanding. A basic model of the current thinking in the field is presented in Figure 1. Hh protein is thought to bind to a receptor called Patched (Ptc), a 12-pass membrane protein with a topology similar to transporters (Hooper and Scott 1989; Stone, Hynes et al. 1996). In anterior cells, Ptc normally suppresses activation of the Hh signaling pathway by inhibiting the activity of another membrane protein, Smoothened (Smo), which has homology to G-protein coupled receptors (Alcedo, Ayzenzon et al. 1996). The mechanism of inhibition is unclear, although it does not seem to be dependent on a stoichiometric interaction between Ptc and Smo (Taipale, Cooper et al. 2002). Hh binding relieves the inhibition of Smo by Ptc, allowing Smo to signal to downstream effectors, as well as causing a change in the subcellular localization of both molecules (Denef, Neubuser et al. 2000). Signal transduction occurs though a complex of four molecules (Stegman, Vallance et al. 2000). These are: a kinesin-related molecule, Costal2 (Cos2), that has an inhibitory role in signal transduction; a kinase, Fused (Fu); Suppressor of Fused (SuFu), a PEST domain containing protein; and Cubitus interruptus (Ci), a Zn-finger transcription factor. Ci has both activator and repressor activities. The full length form of Ci (Ci-155), can be converted into an activator which induces the expression of Hh target genes, while a cleaved form (Ci-75) can repress their expression (Aza-Blanc, Ramirez-Weber et al. 1997). In the absence of Hh, the complex of Fu, Su(Fu), Cos2 and Ci is bound to microtubules. Proteolytic cleavage of Ci generates Ci-75, which can leave the complex and localize in the nucleus, where it represses transcription of Hh targets. How the Hh signal is transduced from Smo to this complex is not entirely understood, but recent work suggests that a direct interaction between Smo and Cos2 may play a role (Lum, Zhang et al. 2003; Ogden, Ascano

et al. 2003). Upon activation of the pathway by Hh binding, both Fu and Cos2 are hyperphosphorylated, and cleavage of Ci is blocked, allowing accumulation of Ci-155. Ci-155 is modified, presumably by phosphorylation, and can be released from the complex to translocate to the nucleus to activate expression of target genes. Also involved in the regulation of Ci are Protein kinase A (Lepage, Cohen et al. 1995) and Supernumerary limbs (Slmb) (Jiang and Struhl 1998), which affect its phosphorylation state and cleavage.

In this research, I used the Drosophila wing imaginal disc as a model system to study Hh signaling and movement. The wing imaginal disc is an epithelial sheet, which is composed of two developmentally distinct compartments: posterior and anterior. These compartments can be distinguished by expression of the transcription factor *engrailed*, which marks the posterior. The border region, where the two compartments adjoin, acts as an organizer that patterns the entire disc. Formation of this organizer is controlled by the activity of Hh, which is produced in the posterior compartment, crosses the border, and signals to cells in the anterior. Activation of the Hh signaling pathway in anterior cells near the compartment border leads to expression of Hh target genes, including *ptc*, *knot*, and *collier*. Another target of Hh in the disc is *dpp*, a TGF-β protein, which is secreted by the anterior border cells and is thought to be the major factor responsible for long range patterning in the disc.

Factors affecting Hedgehog distribution

The mature Hh protein contains two hydrophobic modifications, a palmitic acid moiety which is added to the N-terminus (Pepinsky, Zeng et al. 1998), and a cholesterol moiety which is added in a self-cleavage reaction catalyzed by the C-terminus (Lee, Ekker et al. 1994; Porter, Ekker et al. 1996). These modifications make Hh extremely hydrophobic, and it has a high affinity for cell membranes. Despite its hydrophobicity, Hh is known to signal to

cells up to 10 cell diameters away from the posterior cells that produce it (Chen and Struhl 1996). While much of the developmental requirement for Hh is thought to be through the induction of *dpp*, there is evidence that this region is directly patterned by Hh, and that target genes are expressed in this region in a concentration-dependant manner (Mullor, Calleja et al. 1997; Strigini and Cohen 1997). Movement of Hh away from the posterior compartment is required for proper function, as expression of a membrane tethered form of Hh does not fully rescue Hh activity (Strigini and Cohen 1997). Experiments using Sonic Hedgehog (Shh), one of the three vertebrate homologs, have provided further evidence that Hh acts as a true morphogen. When a Hh-insensitive allele of Ptc is expressed in the neural tube, repression of Hh target genes is observed, demonstrating that Hh acts directly in this tissue even at a distance (Briscoe, Chen et al. 2001). Furthermore, Shh has been found to induce gene expression in a concentration dependent manner (Roelink, Porter et al. 1995). It should be noted, however, that the experiments of Roelink et al. were performed on explants with a truncated form of Shh, which is not cholesterol modified. These activities of Hh raise the question of how such a hydrophobic protein is capable of traveling though the disc tissue to signal to cells away from its expression domain.

In addition to the hydrophobic properties of Hh itself, a number of different proteins affect its movement. The range of Hh is restricted by interaction with Ptc, which is upregulated in response to Hh signaling. Anterior clones of *ptc*$^{-}$ cells allow Hh to move farther from the border than it does in surrounding wild-type tissue (Chen and Struhl 1996). Sequestering of Hh by Ptc is most likely an endocytic process. Hh and Ptc have been shown to co-localize to small particulate structures, the formation of which is dependant on dynamin (Tabata and Kornberg 1994; Han, Belenkaya et al. 2004; Torroja, Gorfinkiel et al. 2004).

These structures have also been observed in mammalian systems (Incardona, Lee et al. 2000). The restriction of Hh movement is functionally important for proper development, as misexpressing Hh, or overexpressing it so that it moves beyond its normal signaling domain, causes misexpression of Hh target genes and severe phenotypes in adults (Porter, Ekker et al. 1996).

Another protein involved in regulating Hh movement is Dispatched (Disp), a large multi-pass transmembrane protein with a topology similar to that of Ptc (Burke, Nellen et al. 1999), as well as to transporter proteins. It is expressed in the posterior compartment of imaginal discs, and is required for proper secretion of fully modified Hh, but not an N-terminal form of Hh that lacks cholesterol modification. A vertebrate homolog of Disp has also been found, and has been shown to have a similar action (Ma, Erkner et al. 2002). The mechanism of Disp function is unknown. It may act directly as a Hh transporter, allowing modified Hh protein to pass though the plasma membrane, or it could be involved in vesicular trafficking of Hh within posterior cells.

Two other proteins with a role in Hh movement are Dally-like (Dlp), a heparin sulfate proteoglycan (HSPG), and Hedgehog interacting protein, or Hip. The first indication that HSPGs had a role in Hh signaling came with the discovery of the gene *tout-velu* (*ttv*) (Bellaiche, The et al. 1998), which shows a *hh*$^{-}$ like phenotype when mutated in flies, and encodes a protein required for proteoglycan modification. Dlp is the major target of Ttv function in flies (Desbordes and Sanson 2003), and it is necessary for the movement of Hh into the anterior compartment. Interestingly, only cholesterol modified Hh seems to require HSPG function; unmodified Hh is able to travel even in the absence of these molecules (The, Bellaiche et al. 1999). Hip is a membrane glycoprotein which has been found to bind to all

three vertebrate Hh proteins (Chuang and McMahon 1999). Its function *in vivo* appears to be to restrict the movement of Hh, and, like Ptc, its expression is upregulated in response to Hh signaling.

There has also been evidence that Hh may form a high molecular weight complex, and that this complex may have a greater solubility and capacity for movement in an aqueous environment than the monomer. Such complexes have been isolated from mouse limb bud (Zeng, Goetz et al. 2001) as well as from cultured cells producing Shh (Chen, Li et al. 2004), and in both cases have been shown to be active. It is unclear whether these complexes are simply Hh multimers, or whether there are other proteins, or perhaps lipids, present. While it is unknown whether such a form exists in flies, the Hh N-terminal fragment has been observed to form a tetramer (J. Knight, personal communication).

We must also consider the possibility that there are substantial differences in the way Hh movement is regulated in vertebrates and flies. Early work in the fly suggested that cholesterol modification was required to limit the range of Hh signaling. Expression of a truncated form (HhN), which does not get cleaved or cholesterol modified, in the normal *hh* expression domain causes a wide expansion of the Wingless (Wg) stripe, a marker for Hh signaling in the embryo. Expression of full length Hh under the same conditions caused only slight increase in Wg levels, and less severe adult phenotypes (Porter, Ekker et al. 1996). A similar result was obtained in the disc, where unprocessed HhN has been shown to be poorly sequestered (Burke, Nellen et al. 1999). In contrast to these results, work in vertebrates, showed that expression of ShhN caused target gene activation in a reduced range when compared to full length Shh (Lewis, Dunn et al. 2001). This discrepancy may be an artifact of the different expression systems used in the experiments, but may also indicate a real

difference between flies and vertebrates. In addition to these differences, Hip is not present in flies at all.

While we have gained some insight into the complex regulation of Hh movement and signaling, the mechanism of movement is not yet clear. The possibility remains that diffusion is the major mechanism by which Hh moves. A multimeric form of Hh could allow it to travel through tissue by burying the cholesterol and palmitoyl groups within a hydrophobic core. Glycoproteins such as Dlp could also aid Hh diffusion, as has been suggested (Han, Belenkaya et al. 2004). Another possible mechanism for Hh movement that has been proposed is transcytosis. As previously mentioned, Hh is found in endocytic structures in the anterior cells of imaginal discs. These structures could be involved in movement of Hh across the epithelial plane, with Hh undergoing endocytosis, traveling through a cell, and being exocytosed on the anterior side. This would avoid the problems of Hh diffusing in the extracellular environment, where it would be retarded by interaction with cell membranes. There is as yet no evidence to support such a model in Hh signaling, but such a phenomenon has been reported to be involved in the transport of Dpp (Entchev, Schwabedissen et al. 2000). An alternative hypothesis is that direct contact between Hh source cells and Hh receiving cells is required for movement of the Hh signal. The imaginal disc is an epithelium of closely packed cells that form tight junctions, and Hh could conceivably travel from cell to cell across their closely juxtaposed membranes. Alternatively, long filopodial extensions, or cytonemes, could be involved in the transport of Hh. Cytonemes are polarized extensions that are produced by cells in the wing disc, and extend towards the organizer at the compartment border (Ramirez-Weber and Kornberg 1999). While the function of these extensions is not

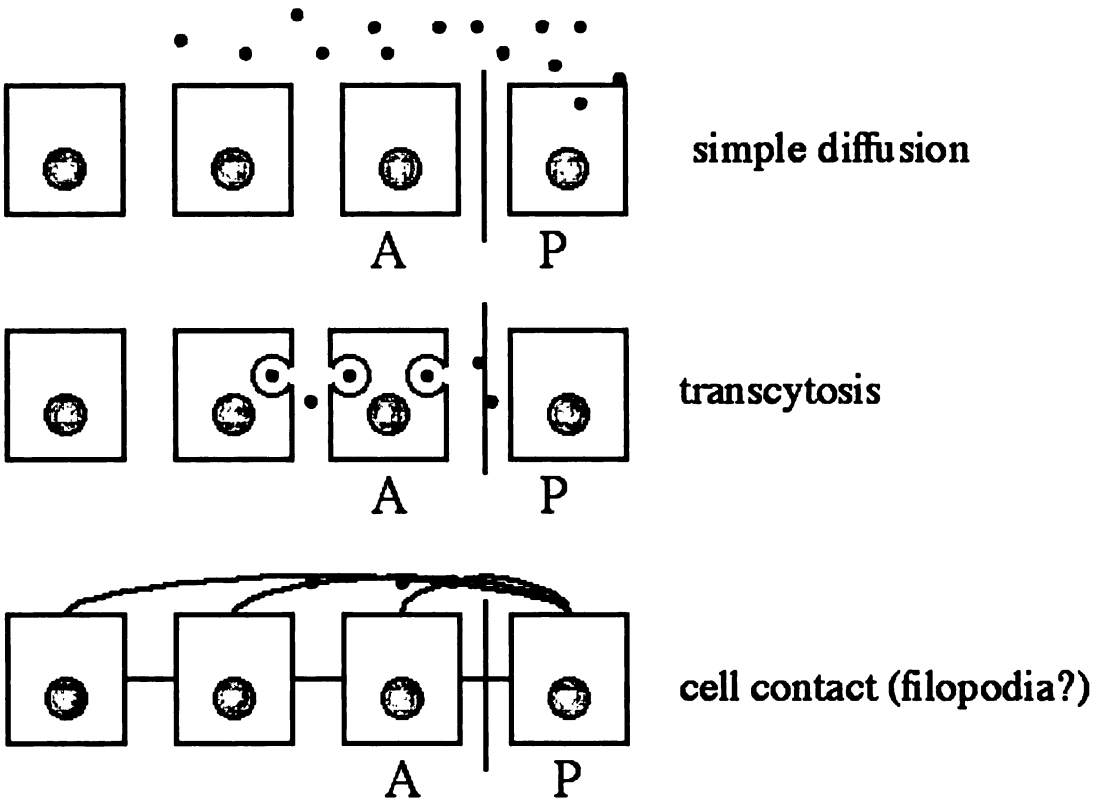


Figure 2. Possible mechanisms of Hh movement

yet understood, they have been proposed to transport developmental signals back to the cells that make them.

In this work I use several different approaches to investigate how Hh moves, and how its signal is received by the cells that respond to it. In Chapter One, I describe a cell culture based system that I developed to examine the requirements for paracrine signaling by Hh. Paracrine signaling is signaling between cells, as opposed to autocrine signaling, which is the reception of signal by the cell that makes it. The data provides evidence for a cell-contact based mechanism of Hh transport. Chapter Two describes an investigation into the role of endocytosis in Hh signaling. Using a construct that overexpresses a conditional mutant of *shibire*, the *Drosophila* dynamin, I show that Hh movement into the anterior compartment is not blocked by defects in endocytosis. I also describe experiments that examine the role of endocytosis in Hh pathway activation. In Chapter Three, I describe experiments that characterize the role that hydrophobic modifications play in Hh signaling in *Drosophila*. I used both *in vivo* and cell culture assays to observe the effects of removing the N-terminal palmitoyl and C-terminal cholesterol modifications on Hh localization and signaling. My studies together provide insight into how Hh protein travels through the disc and signals the appropriate cells in order to properly pattern the disc.

Chapter 2

Cell-cell contact in paracrine signaling by Hedgehog

Introduction

In this chapter the requirements of paracrine signaling by Hh are examined in order to distinguish between the various models proposed for the movement of active Hh protein. In particular, the role of cell-cell contact in Hh movement is probed. I use several different cell culture based assays to answer the question of whether the Hh signal is capable of free diffusion, despite its tight membrane localization. If the Hh molecule is able to move by diffusion, paracrine signaling should take place even without direct contact between the Hh source and receiving cells. If, on the other hand, Hh moves through a cell contact based mechanism, signaling will only take place when the two cells are allowed to touch.

The *Drosophila* S2 cell culture system is well suited for answering this question, because the cells are easily manipulated, and paracrine and autocrine signaling can be distinguished by physically separating Hh producing cells and naive cells. In addition, these cells do not normally produce Hh, although they are capable of responding to it. This allows the properties of different forms of Hh, for instance cholesterol modified and unmodified, to be assayed individually. One caveat of using this system, however, is that it may be difficult to relate the results directly to the *in vivo* situation.

Previously, there has been no demonstration of paracrine signaling by fully modified Hh in cultured *Drosophila* cells. In contrast, paracrine signaling by Shh has been observed in mammalian systems (Zeng, Goetz et al. 2001; Chen, Li et al. 2004). However, these

experiments were performed with highly purified, concentrated fractions, and it is unclear whether they represent physiologically relevant concentrations of Hh, or whether their findings are applicable to *Drosophila*.

I found that fully modified Hh is capable of signaling to naive cells only when cell-cell contact is allowed. When cells are prevented from forming contacts, either by physical separation or by agitation, paracrine signaling by fully modified Hh is blocked. However, autocrine signaling and signaling by unmodified Hh are unaffected. I also examine uptake of Hh by receiving cells in this cell culture system, and investigated whether uptake of Hh correlates with signaling. Taken together, my results argue against models for Hh movement that rely on free diffusion, and are consistent with a cell contact based mechanism for Hh transport.

Results

Requirements for paracrine Hh signaling in cultured cells. These experiments used two different Hh expression constructs (Figure 1D): one expressing a full-length Hh protein (Hh-FL) that is processed normally to produce cholesterol modified Hh (HhNp), and a truncated protein which produces unmodified Hh (HhN). In both versions the N-terminal palmitoylation signal was intact. Figure 2E shows a Western blot of lysate and conditioned media from cells expressing each of the constructs, probed with a polyclonal α-Hh antibody which recognizes epitopes from both the N- and C-terminal halves of the protein. In a lysate from Hh-FL cells, four separate forms of Hh were present: a band near 50kD, which is the size of the uncleaved Hh, a 25kD band representing C-terminal Hh, a 19kD band representing the Hh N-terminus, and a small molecular weight band (~9kD), which is most

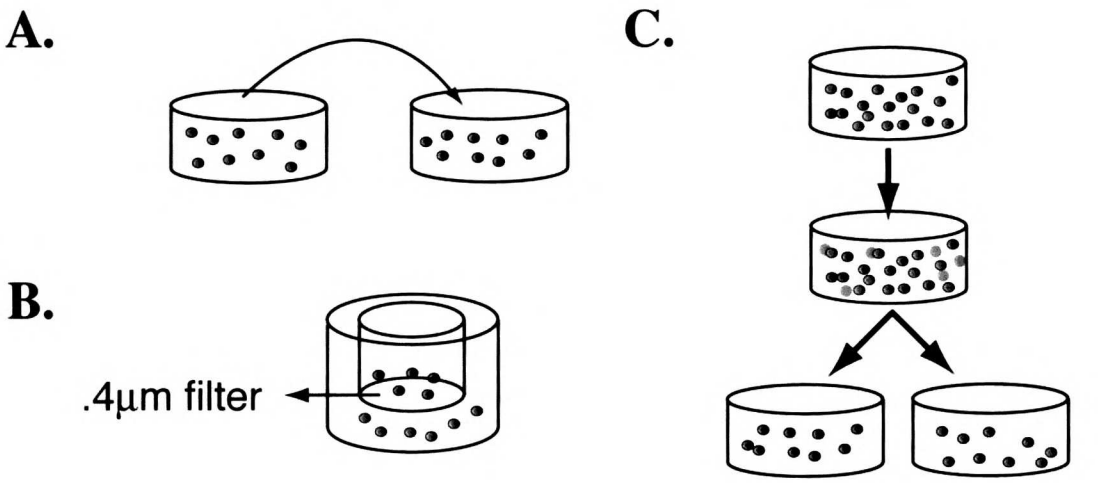


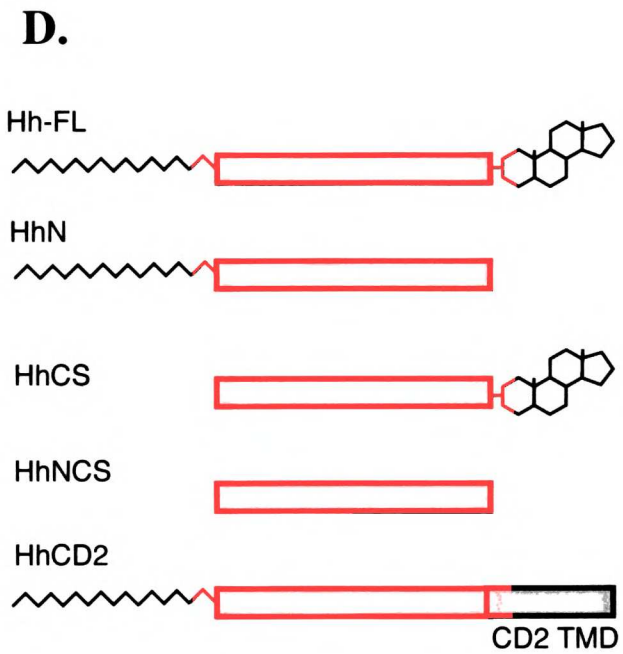
Figure 1. Experimental design and constructs used

A) Conditioned media from Hh expressing cells is incubated with naive cells

B) Cells are cultured with the Hh expressing and naive cells separated by a 0.4 micron filter

C) Cells expressing Hh and CD2 are co-cultured with naive cells, incubated with α-CD2 beads, then subjected to magnetic separation

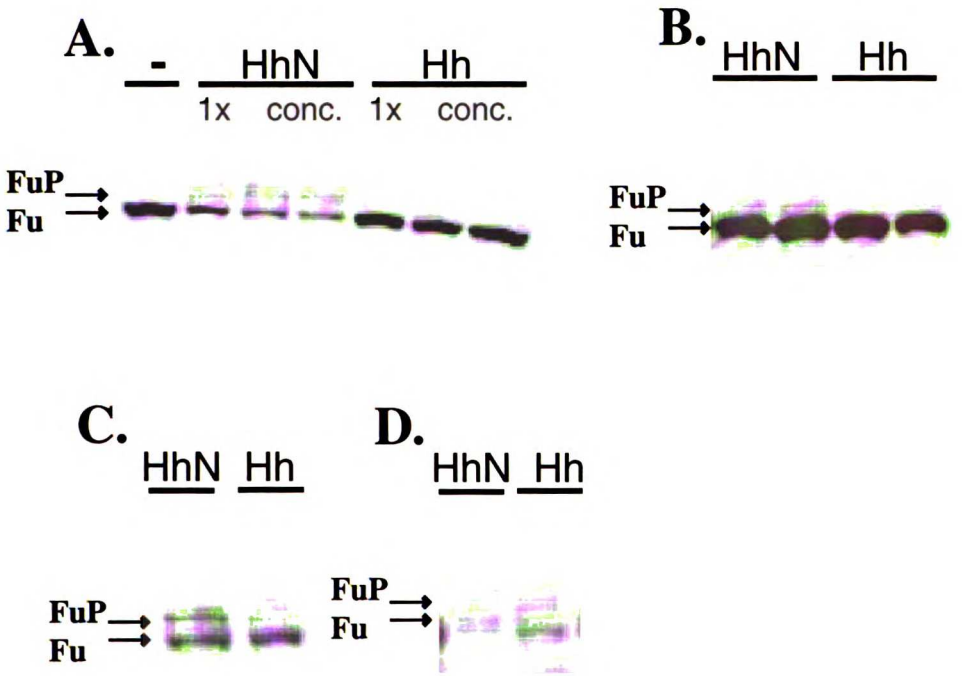
D) Proteins produced by the constructs used in this study



likely a C-terminal degradation product. As expected, in lysate from HhN cells only the 19kD band was present.

Hh signaling was assayed using Western blots to observe the phosphorylation state of the Fu kinase. When a lysate from unstimulated cells was fractionated on an SDS-PAGE gel and probed for Fu, a single band was present, representing the basal phosphorylation state of Fu. However, in cells responding to Hh, a portion of this Fu migrated at a slower rate. This band represents a hyperphosphorylated form of Fu, FuP (Therond, Knight et al. 1996). Lysate from cells expressing either Hh-FL or HhN contained both Fu and FuP, demonstrating that both modified and unmodified Hh are capable of autocrine signaling (Figure 2D).

This result, however, does not address the question of whether both Hh-FL and HhN are capable of paracrine signaling. I developed three different, cell-culture based assays for paracrine Hh signaling. These are diagrammed in Figure 1. For the conditioned media assay (Figure 1A), populations of cells expressing either Hh-FL or HhN were incubated in Schneider media for several days, then the media was collected, subjected to centrifugation and filtered to remove cells. This conditioned media was diluted 1:5 with fresh S2 media, and used to treat naive, untransfected, S2 cells. Results from this assay are shown in Fig. 2A. After incubation of naive cells in HhN conditioned media, both Fu and FuP were present, indicating an activated Hh signaling pathway (lane 2). However, treatment with media conditioned by cells producing Hh-FL did not activate Hh signaling, as only the faster migrating form of Fu was present (lane 5). Lysate from naive S2 cells, containing only this basally phosphorylated Fu form, is shown as a control (lane 1).



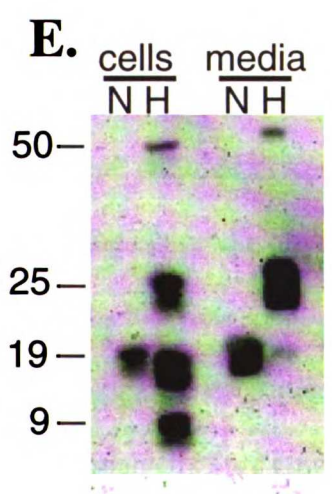


Figure 2. Hedgehog signaling in paracrine assays. All lanes in A-D are lyates from naive cells after incubation in the various experiments. Lanes in E are lyates from Hh expressing cells. N indicates cells incubated with HhN cells or conditioned media, H indicates cells incubated with Hh-FL cells or media. A-D are probed with α-Fu, E wih α-Hh.
A) conditioned media assay
B) filter assay
C) co-culture assay
D) Hh cell lysate
E) Hh produced by HhN or Hh-FL cells

One possible explanation for this result is that HhNp is simply not present at sufficiently high levels in the media conditioned by cells expressing Hh-FL promote signaling. Although this media contains some of the modified N-terminal fragment, levels are considerably lower than that of the unmodified N-fragment present in HhN conditioned media (Figure 2E). To address this possibility, the conditioned media was concentrated using centricon filters. Media was concentrated 5-fold, then diluted at a 1:1 ratio with fresh media, resulting in a final concentration that was 13.5x the concentration of conditioned media used in the original experiment. However, this media also had no effect on the naive cells, as only a single Fu species was present (Figure 2A, lanes 6 and 7). The concentrated media itself did not inhibit signaling, as the concentrated HhN media signaled effectively (lanes 3 and 4).

While this concentration was not sufficient to allow paracrine signaling by HhNp, it could be that the levels of Hh immediately surrounding producing cells is even higher than that used, and that cells that are very close can signal even without making contact. To examine this possibility, Hh expressing and naive cells were cultured in the same well, but prevented from contacting each other. This experiment is diagramed in Figure 1B. The two populations were cultured in a single well, but separated by a porous filter. This filter was inverted, and the Hh producing cells were seeded on the bottom side and allowed to settle. After these cells had time to adhere, the naive cells were seeded on the upper side of the filter, and the apparatus was placed in a well of fresh S2 media. This method results in two populations of cells that are cultured adjacent to each other, with only the filter itself separating them. The 0.4 micron pore size used was large enough for passage of large molecules, but should prevent contact between the cell bodies. After overnight incubation, the naive cells were removed, lysed, fractionated on an SDS-PAGE gel, and assayed for the

presence of FuP. The results from this experiment, shown in Figure 2B, are essentially the same as in the previous experiments: the HhN expressing cells were capable of activating the signaling pathway in naive cells, but those expressing Hh-FL are not.

Finally, cells were co-cultured with no barriers to determine if paracrine signaling could occur when cells were able to contact each other. For this experiment, diagramed in Fig. 1C, cells were co-transfected with either Hh-FL or HhN, and the membrane protein CD2. After recovering for several days, the cells were cultured with naive cells overnight, then magnetic beads coated with antibody to CD2 were added, and the cells were incubated for 30 minutes to allow binding of CD2+ cells to the beads. Application of a magnetic field removed the CD2+ cells, which are also Hh+, leaving a purified population of naive cells in the supernatant. This naive cell population was assayed for purity by immunostaining with α-Hh. No cells were observed that had the strong staining associated with Hh expression. In this experiment, unlike in the previous ones, FuP was seen in the naive cells incubated with Hh-FL expressing cells (Fig 2C). This result suggests that allowing contact between Hh expressing and non-expressing cells is necessary and sufficient for signaling to take place.

Signaling occurs using a membrane bound form of Hedgehog, but is blocked when cell-cell interaction is inhibited. To confirm this model, it is important to establish that the two populations are in fact contacting each other. To accomplish this, a construct that expresses a fusion protein of Hh and the CD2 transmembrane domain was used (HhCD2, shown in Figure 1D). This fusion results in a membrane-bound form of Hh, and has previously been shown to be active *in vivo* (Strigini and Cohen 1997). Cells were co-transfected with HhCD2 and CD2, co-cultured with naive cells, and separated as described above. As shown in Fig. 3A, HhCD2 was capable inducing naive cells to produce FuP,

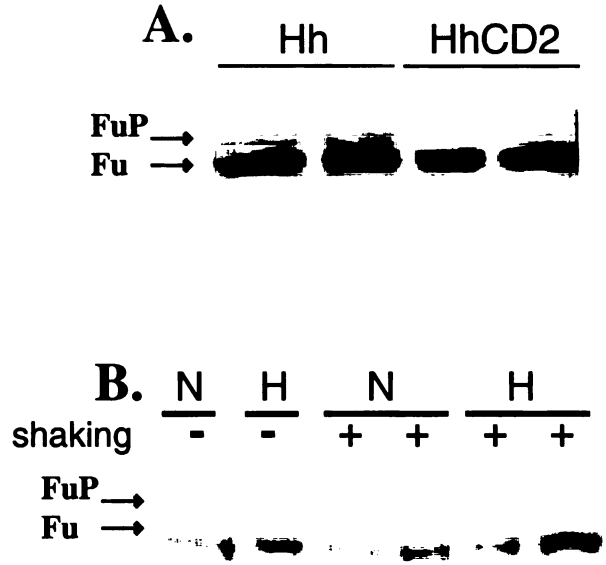


Figure 3. Cell contact in Hh signaling
A) Fu western of cells responding to co-cultured HhCD2 expressing cells
B) Fu phosphorylation was blocked in cells co-cultured while shaking

indicating that Hh signaling has occurred. HhCD2 did seem to be slightly less active, as the levels of FuP were slightly less than those produced by co-culture with Hh-FL cells. The reason for this reduced activity is unclear, although it is reproducible. HhCD2 seems to have reduced activity in autocrine signaling as well, so it may be that the membrane bound form has a confirmation that is less than optimal.

If cell-cell contact really is required for signaling by Hh-FL, one would predict that inhibition of contact would block signaling. To test this, Hh producing and naive cells were co-cultured in cultures that were agitated to disrupt the formation of stable contacts between cells. For this experiment, cells co-transfected with CD2 and either Hh-FL or HhN were mixed with naive cells, and the cultures were shaken at 500 rpm throughout the incubation period. This speed was sufficient to vastly reduce adherence of cells to each other as well as to the bottom of the wells, as monitored by visual inspection. After overnight incubation, cells were separated magnetically, as previously described, and their Fu phosphorylation was assayed. This shaking blocked paracrine signaling by the Hh-FL cells (Fig. 3B), although signaling by HhN cells was unaffected, suggesting that it is presentation of Hh to its receptor, and not a later step in the pathway, that is affected.

Increasing Dispatched expression in S2 cells does not increase secretion of Hh. In vertebrate systems, expression of Disp has been shown to increase the level of active, modified Hh that is secreted into the media by cultured cells (Ma, Erkner et al. 2002; Tian, Jeong et al. 2004). To see whether expressing Disp could allow secretion of active Hh by S2 cells, Hh-FL and Disp were co-expressed in S2 cells. The cells were cultured for several days, then the cells and conditioned media were harvested, and the cells lysed. Samples were run on an SDS-PAGE gel, and accumulation of the processed N-terminal fragment was

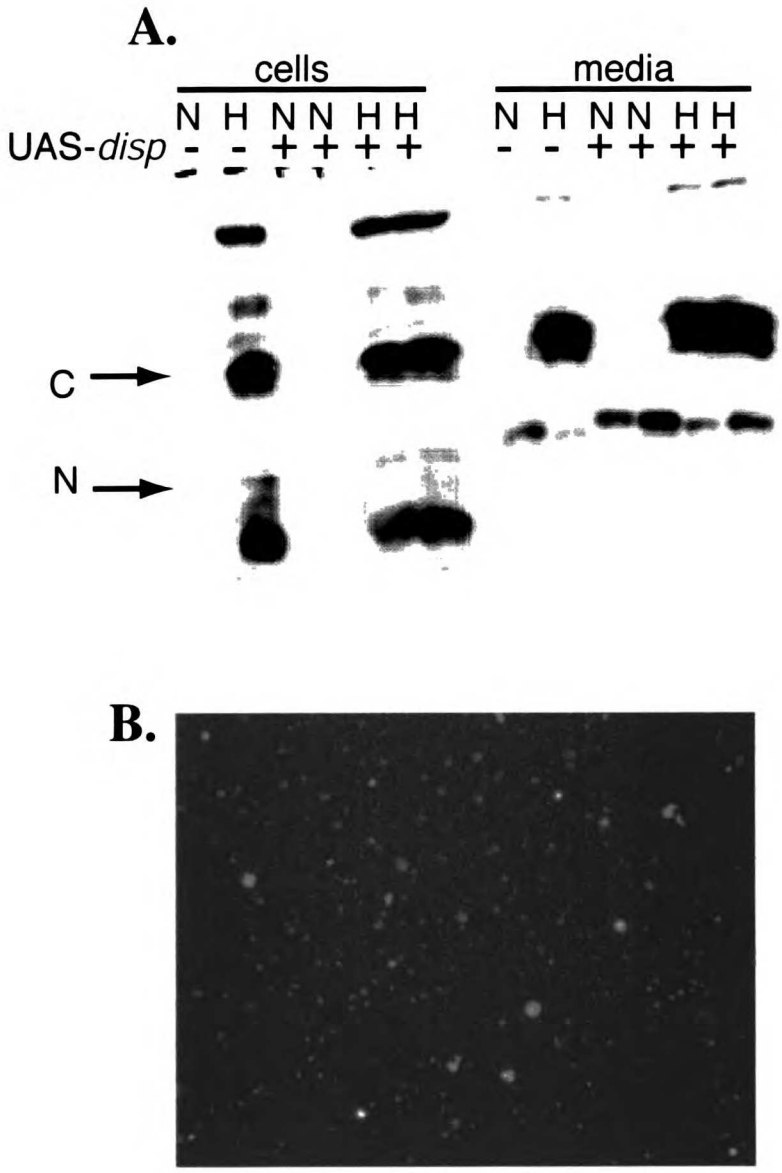


Figure 4. Hh secretion in cells overexpressing Disp
A) α-Hh immunoblot of cells and conditioned media. The levels of HhNp in S2 media do not increase when Disp is expressed
B) α-HA immunostaining of cells transfected with HA tagged *disp*. Cells stained red are expressing Disp.

observed using a Western blot for Hh. The results can be seen in Figure 4A. No difference was seen in levels of HhNp plus or minus Disp. HhN expressing cells, which should not be affected by the presence of Disp, are included as a control. To ensure that the Disp protein was being expressed, a form of Disp tagged with HA was used, and transfected cells were stained with α-HA. Levels of expression of this Disp-HA fusion were consistent with expression of Hh after transfection (Fig. 4B). Based on these results, I argue that the capacity of S2 cells to secrete Hh is not limited by the level of Disp, and that the absence of Hh activity in the concentrated media was not due to the absence of Disp.

Endocytosis of Hh protein occurs both *in vivo* and in culture. In the wing imaginal disc, Hh protein is distributed in a distinctive pattern. Staining for Hh protein by the α-Hh antibody revealed that in the posterior compartment Hh protein is present in a diffuse pattern, but in the anterior compartment most of the Hh is present in small, particulate structures. These particles are likely to be endocytic in nature, as their formation is blocked in dynamin mutants (Tabata and Kornberg 1994). Figure 5 is a confocal image of the border region of an imaginal disc, stained for Hh. Both types of Hh distribution were present. The functional significance of this uptake of Hh by anterior cells remains unclear. I examined uptake in S2 cells exposed to Hh. S2 cells normally do not express Hh, and untreated cells did not stain with the α-Hh antibody (Fig. 6A). However, after co-culture with Hh expressing cells, particles of bright staining, presumably endocytic structures, were present (Fig. 6B). Surprisingly, similar structures were present in cells treated with Hh-FL conditioned media, although these cells do not activate their Hh signaling pathways (Fig. 6C). Cells treated with HhN conditioned media, which do respond to Hh, also contained Hh particles, but they tended to be fewer in number.

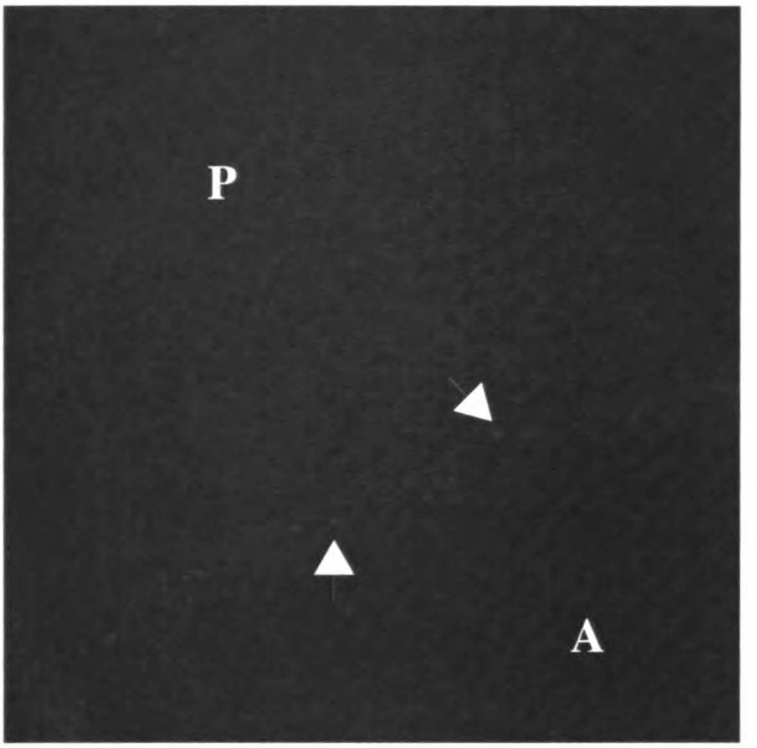


Figure 5. Hh uptake in discs
confocal image of 3rd instar wing disc stained with a-Hh. Posterior and anterior compartments are marked. Note the punctate staining at the border (arrowheads).

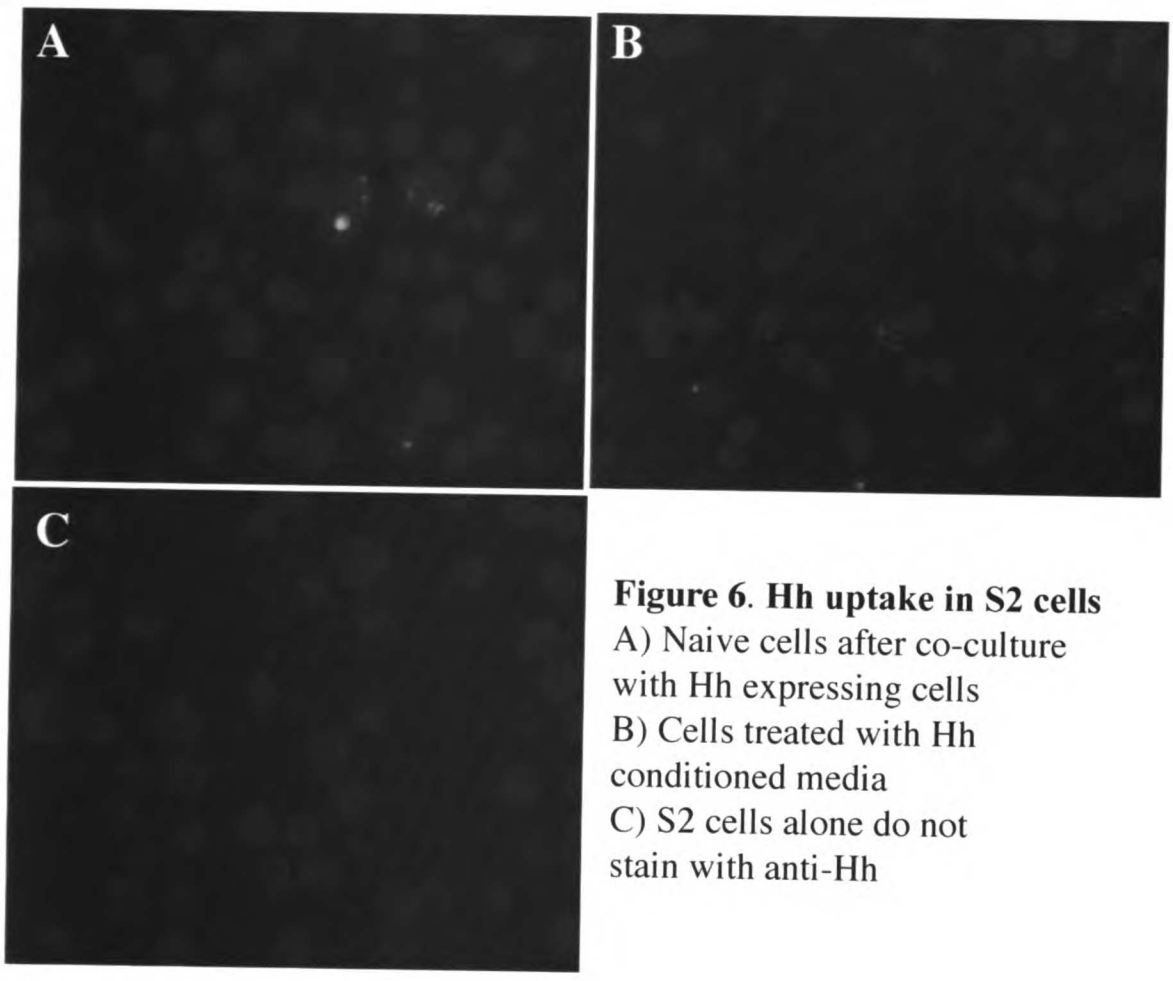


Figure 6. Hh uptake in S2 cells
A) Naive cells after co-culture with Hh expressing cells
B) Cells treated with Hh conditioned media
C) S2 cells alone do not stain with anti-Hh

The presence of Hh staining in cells does not correlate with Hh signaling, but the uptake of HhNp does. These results imply that uptake of Hh cannot be correlated with active signaling. However, the Hh antibody used was polyclonal and recognizes the entire Hh molecule, making it impossible to determine if cells in the two conditions are taking up the same forms of the protein. To resolve this question, α-Hh Western blots were performed on lysates prepared from the naive cells in each assay. Results are shown in Figure 7. Figures 7A and 7B show lysate and conditioned media from cells producing Hh-FL and HhN. Conditioned media from cells producing Hh-FL contained both the N- and C-terminal fragments; however, in cells treated with this media (Fig. 7C) only the C-terminal form was found. In contrast, lysate from S2 cells co-cultured with Hh producing cells, after separation by magnetic sorting, did contain N-terminal Hh, which correlates with the active Hh signaling pathway in these cells (Fig. 7D). It is important to remember, however, that this is only a correlation and does not necessarily indicate a functional relationship between signaling and uptake. What this experiment does show is that cell contact is necessary not only for signaling, but for the active form of Hh to move to receiving cells. The HhNp present in the Hh-FL conditioned media seems to be inactive, implying that modified Hh can only signal and be taken up by cells when presented in the context of a cell membrane.

Uptake of C-terminal Hh (HhC) in the conditioned media treated cells was unexpected. To examine the specificity of this phenomenon, the uptake of HhC was compared to the uptake of a control protein, a secreted form of GFP. I found that the secreted GFP did not accumulate in S2 cells, supporting the conclusion that the uptake of HhC was specific. Furthermore, uptake of HhC was not affected by the presence of the N-terminal half of the molecule, as it was seen in cells treated with conditioned media that contained only C-

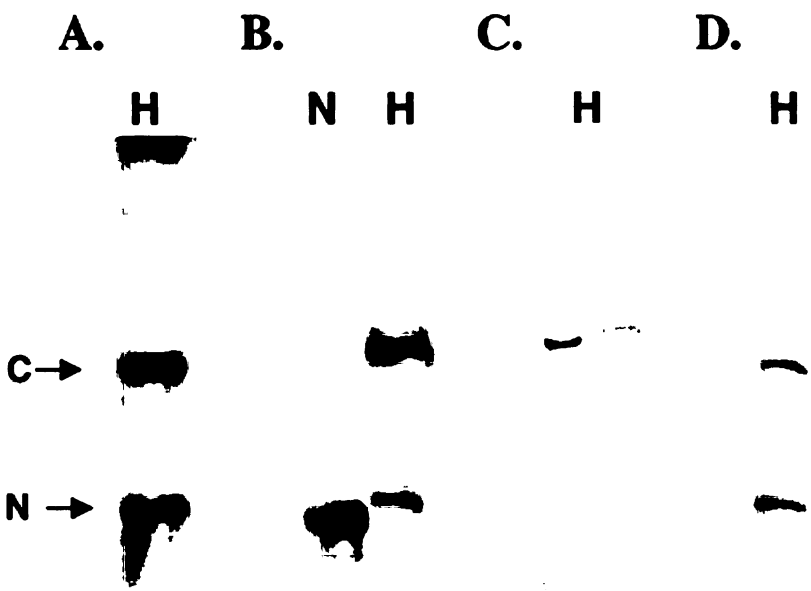


Figure 7. Uptake of N and C regions of Hh protein in S2 cells
α-Hh western, arrows show expected size of Hh fragments. Experiments using HhN and Hh-FL are marked with N and H, respectively
A) Lysate from Hh expressing cells
B) Conditioned media from Hh expressing cells
C) Naive cells after treament with Hh conditioned media
D) Naive cells after co-culture with Hh expressing cells

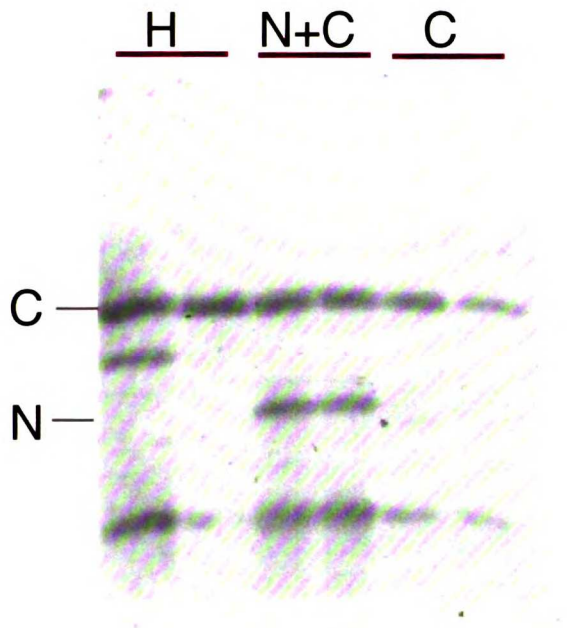


Figure 8. Uptake of Hh C-terminus in S2 cells.
S2 cells treated with conditioned media from cells expressing either full-length Hh, The N and C-terminal fragments separately,or the C-terminus alone. The Hh C-terminus was taken up in each case.

terminus (Fig. 8). However, expression of HhC in flies had no apparent phenotype, implying that HhC has no role following its proteolytic removal from the N-terminus. The mechanism and possible function of this uptake remain unclear.

Discussion

In recent years evidence has been mounting that mechanisms other than simple diffusion play a role in movement of signaling proteins. Association of proteins with heparin sulfate proteoglycans has been shown for molecules such as FGF and Wg, and genetic experiments suggest a link between these proteins and Hh signaling as well. In the case of Hh, an impressive number of proteins that affect movement have been discovered, including Disp, Skinny hedgehog (Ski), the Hh acetyltransferase, and its own receptor, Ptc. These discoveries argue for a highly regulated process involved in moving Hh to the cells that must respond to it. Furthermore, the dual hydrophobic modifications of Hh, as well as its strong affinity for membranes, are likely to retard its passage through the extracellular environment. In spite of this, simple diffusion has not been ruled out as the major mechanism of Hh movement.

My data is consistent with the idea that simple diffusion is insufficient to explain Hh movement. Fully modified Hh was unable to signal to receiving cells even when in close proximity, unless cell-cell contact was allowed. Further evidence of close contacts between cells producing and cells receiving Hh signal was found by observing signaling with a membrane-bound Hh, as well as by the ability of co-cultured cells to take up a fully processed Hh N-terminal fragment. While it is not clear whether uptake is required for signaling to occur, a close association between the expressing and responding cells seems to

be required. Importantly, no N-terminal Hh was found in naive cells after treatment with Hh-FL conditioned media.

Recent data from vertebrates has indicated that an active form of Hh can be isolated from limb buds or Hh expressing cell lines (Zeng, Goetz et al. 2001; Chen, Li et al. 2004). This form is fully processed and multimeric, which may allow it to reduce its hydrophobicity by burying the cholesterol and palmitoyl groups in the core of the complex. This vertebrate complex has a high molecular weight, and could represent a particle that contains multiple proteins and lipids. The apparent discrepancy between these results and my work may be explained by differences between fly and vertebrate systems, or they may be a result of using highly concentrated protein that does not mimic normal physiological conditions.

Although my results argue against simple diffusion as a mechanism for Hh movement, they do not answer the question of what sort of cell contacts may be utilized to allow Hh signaling to occur *in vivo*. Transcytosis remains a formal possibility, as close contact could be required to pass protein from cell to cell. Alternatively, Hh could diffuse across plasma membranes using direct contacts between neighboring epithelial cells to pass from cell to cell. Cytonemes, which are known to be produced by cells at a distance from the border, have been proposed as a possible mechanism for the reception of morphogen signal, although how their mechanism of action is unclear. Whether or not cytonemes are involved, paracrine signaling clearly requires direct contact with Hh containing membranes, perhaps in an active process that requires the formation of a stable contact, and results in the physical transfer of Hh protein.

Materials and Methods

Cell culture: *Drosophila* S2 cells were cultured in Schneider media (Invitrogen), or in M3 media (Sigma), both supplemented by 10% FCS. All cell culture experiments were performed at 24°C. Transfections were performed using a Qiagen effectene kit, following the manufacturer's protocol. For conditioned media experiments, 5-6 day cultures were spun down at 700 rpm, then the supernatant was passed through a 0.2μm filter to remove any cells not spun down. Conditioned media was concentrated by centrifugation over a Centricon filter with a 10,000 kD cutoff. Filter experiments used transwell polycarbonate inserts, with a 0.4μm pore size (Costar). Cells were incubated overnight in both cases. For the co-culture experiments, cells were transiently transfected with DNA encoding rat CD2, actin-GAL4 (to drive expression) and either UAS-*hh* or UAS-*hhN* (Ramirez-Weber, Casso et al. 2000). After 2-3 days, cells were recovered, mixed with naive S2 cells, and cultured overnight. Mixed cell populations were spun down gently, and resuspended in PBS with 2%FBS before adding α-CD2 beads (Dynal), then incubated on a rotator for 30 minutes at 4°C. Separations were performed using a magnetic separator, also from Dynal.

Western blotting: All cells were lysed in NP-40 buffer (150mM NaCl, 10mM Sodium Phosphate buffer (pH 7.2), 1% NP40) plus DNase (50μg/ml) and RNase (25μg/ml). Blots were probed with rabbit α-Hh at 1:10,000 (Tabata and Kornberg 1994) or rabbit α-Fu at 1:10,000 (Therond, Alves et al. 1996), then with the secondary antibody, HRP α-rabbit (Jackson).

Immunostaining: Imaginal discs were fixed and stained as previously reported (Tabata and Kornberg 1994). Cells were transferred to a glass chamber slide (Nalge Nunc), fixed in 4%

Chapter 3

Role of endocytosis in Hh movement and signaling

Introduction

When Hh protein distribution was first examined, it was observed that Hh was largely diffuse in the posterior compartment, but in the anterior compartment was primarily localized to small, particulate structures (Tabata and Kornberg 1994). These structures co-localized with Ptc, the Hh receptor, and are believed to be endocytic in nature, since Ptc has been shown to be in multi-vesicular bodies (Capdevila, Pariente et al. 1994). Similar Shh containing structures have been observed in vertebrate cells (Incardona, Lee et al. 2000). The functional relevance of Hh endocytosis is not yet understood.

Endosomal trafficking and signaling by cell-surface receptors are both highly regulated processes with important roles in regulating the relationship between a cell and its environment. It is not surprising, therefore, that endosomal transport can affect the activities of signaling pathways. Many receptors are endocytosed in a ligand-dependant manner, and for many of these receptors, including members of the receptor tyrosine kinase (RTK) family, endocytosis after ligand binding serves at least in part to down-regulate the receptor (Stoscheck and Carpenter 1984). After endocytosis, receptors can be targeted to the lysosome for degradation, or separated from ligand and recycled to the surface. However, this downregulation is not the only way that endocytosis can be involved in interactions between ligand and receptor. More recently, endocytosis has been proposed to play a role in the distribution of ligand, particularly in developmental contexts. Experiments with a GFP

labeled Dpp have shown that long-range Dpp movement is blocked when endocytosis is disrupted (Entchev, Schwabedissen et al. 2000), suggesting that Dpp may move via a transcytosis based mechanism. The distribution of Wg protein is also dependent on endocytosis, although evidence in this case suggests a role in removing extracellular Wg, rather than in promoting movement (Strigini and Cohen 2000). Even more intriguing is the Notch pathway, where there seems to be a requirement for endocytosis of the Notch ligand, Delta, to fully activate the pathway (Parks, Klueg et al. 2000).

Several possible roles for the role of endocytosis in Hh signaling have been proposed. Endocytosis could be directly involved in signal transduction, perhaps by targeting Ptc to an intracellular compartment where it is sequestered and prevented from inhibiting Smo. Supporting this idea is data that indicates that both Ptc and Smo alter their subcellular localization on Hh binding (Denef, Neubuser et al. 2000; Incardona, Gruenberg et al. 2002), as well as my own experiments that reveal a correlation between uptake and signaling in cultured cells. Alternatively, receptor mediated endocytosis of Hh could downregulate signaling. A recent screen for mutants that affect Hh signaling in vertebrates found that loss of function of Rab23, a vesicular transport protein, increased Hh signaling. This suggests that Rab23 is a negative regulator of Hh signaling (Eggenschwiler, Espinoza et al. 2001).

Another possibility is that endocytosis is involved in Hh transport, either promoting Hh transport through transcytosis, or restricting Hh movement by sequestering the protein. It is known that Ptc is involved in sequestering Hh and limiting its movement (Chen and Struhl 1996), and it may be that targeting to the endocytic pathway is part of this process. Of course, it is also possible that Hh endocytosis plays more than one role. It could be involved in both signaling and sequestration, for example.

In this study I examined the role of endocytosis of Hh in the imaginal disc, using a construct that overexpresses a temperature-sensitive form of Shibire (Shi). I examined the effect on Hh localization, and characterize the accumulation of Hh protein in the anterior compartment. I also attempt to determine if endocytosis of Hh is involved in signal transduction, using Ci stabilization to observe pathway activation in the disc, as well as observing phenotypes of adult wings from animals in which Shi has been inactivated during development. My results *in vivo* suggest that uptake of Hh is important for the sequestering and degradation of Hh, but not for signaling or for movement of Hh into the anterior compartment.

Results

Blocking uptake leads to accumulation of Hh in the anterior compartment. To examine the role of endocytosis in Hh localization, a transgenic fly line, which expresses a temperature sensitive form of Shi under control of the Gal4-UAS system, was used. The mutant Shi protein, when overexpressed, acts as a dominant negative and disrupts the action of wild-type Shi. The advantage of using this system is that it is possible to control both the spatial expression of the mutant protein and the timing of Shi inactivation. This helps to reduce the secondary effects of the *shi* mutation, which is cell lethal. Larvae expressing UAS-shi^{ts} under control of various drivers were raised at the permissive temperature, then transiently shifted to the non-permissive temperature (32°C). After incubation, the larvae were dissected and their discs were fixed and stained with α-Hh to observe Hh localization. When Shi^{ts} was expressed using *ptc*-Gal4, which drives expression weakly in the in the entire anterior but most strongly in the anterior border cells, an accumulation of Hh in a stripe near

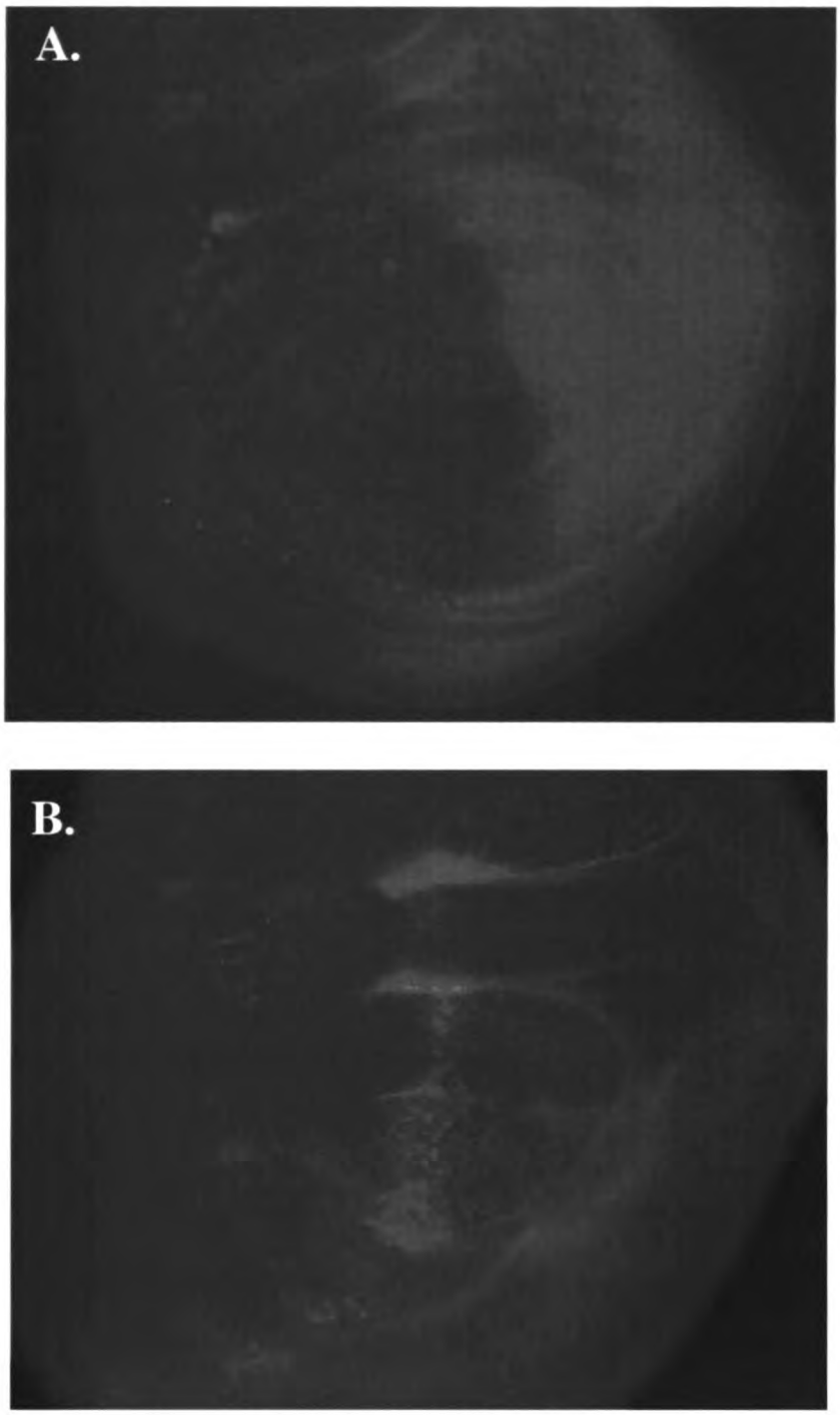


Figure 1. Hh localization in wild-type and *shi* mutant disks
A) wt disc stained with α-Hh, anterior is to the left
B) *ptc*-Gal4; UAS-*shi*^{ts} disc after 20h at 31°C

the border was observed (Figure 1). This accumulation was already evident after six hours, and persisted through at least 24 hours.

I next wanted to determine where this Hh accumulation occurs. If Hh accumulates in posterior, it would imply that endocytosis is required for movement of Hh in the anterior compartment, supporting the transcytosis model. If, on the other hand, the Hh accumulation is in the anterior compartment, it would support the model that endocytosis is involved in Hh sequestration, or in reducing the levels of Hh that reach receiving cells. To address this question I compared the localization of Hh to that of Ci. Ci is evenly expressed throughout the anterior, but full length Ci-155 is present in high levels only in the region near the A/P compartment border, where Hh signaling is most active. Ci-155 was monitored in these discs with the monoclonal antibody 2A1, which recognizes the C-terminus. When discs from larvae raised at the permissive temperature were stained, no apparent overlap between the Hh and Ci localization was observed (Figure 2A). In contrast, high levels of Hh overlapped with Ci in the anterior compartment of *ptc*-Gal4; UAS-*shi*^{ts} discs that had been shifted to the non-permissive temperature (Fig. 2 B).

Part of the stripe of high Hh levels extended posterior to the 2A1 staining. To determine whether this represents Hh accumulation in the posterior, I examined the localization of Hh in relation to Ptc, which is present in discs at high levels right up to the compartment border (Fig 3A). Accumulated Hh in *ptc*-Gal4; UAS-*shi*^{ts} discs, after incubation at the non-permissive temperature, exactly co-localized with Ptc at posterior edge of the stripe, indicating that it was entirely in the anterior compartment (Fig. 3B). Note that the co-localization was quite tight even at higher magnifications (Fig. 4), which is not surprising if the increased Hh levels represent Hh that was bound to Ptc and failed to be internalized.

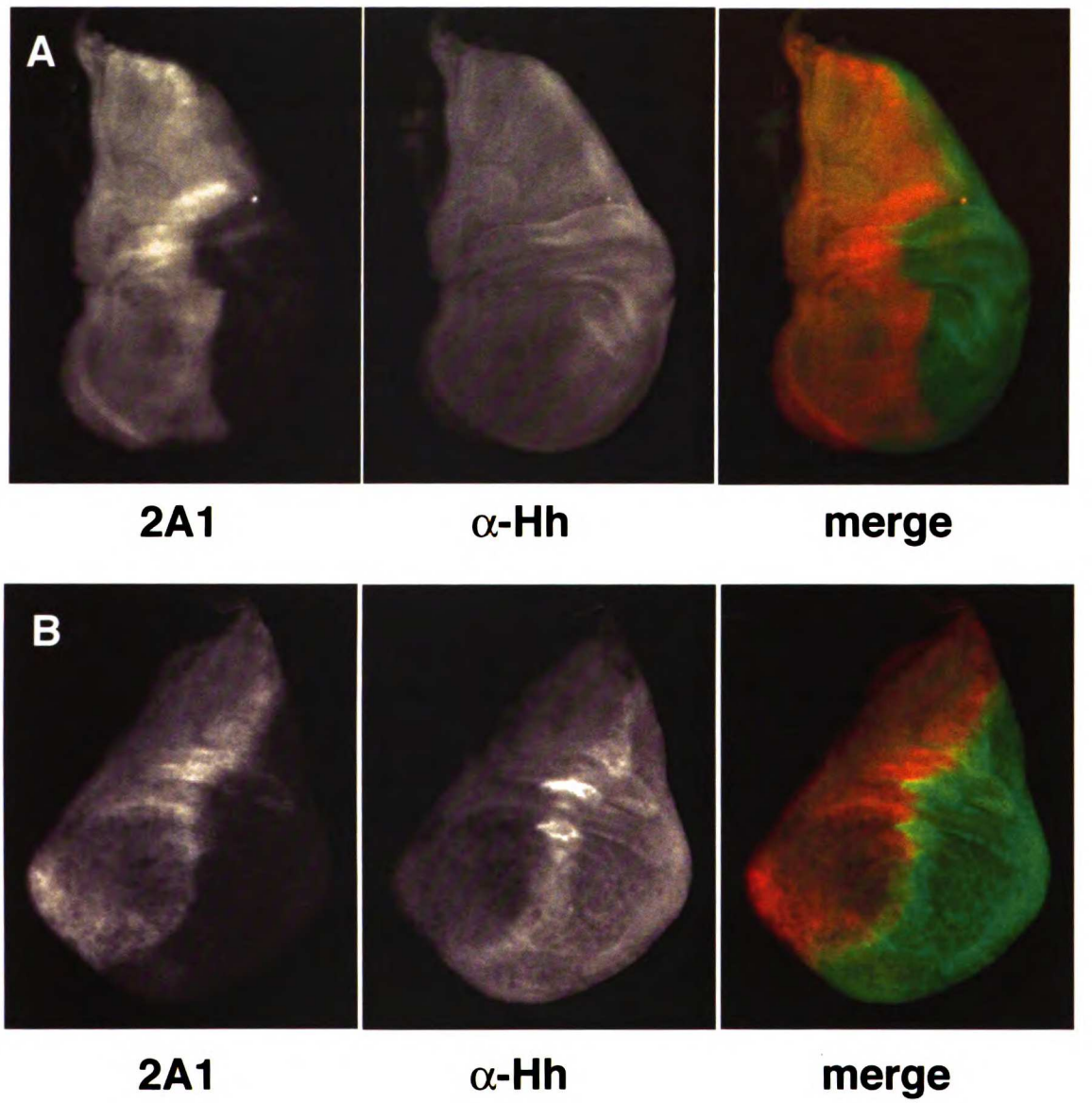


Figure 2. Hh and full-length Ci in discs expressing mutant Shibire
A) *ptc*-Gal4/+;UAS-shi^{ts}/+ discs that have been raised at the permissive temperateure. B) *ptc*-Gal4/+;UAS-shi^{ts}/+ discs that have been held at 32°C overnight. For both discs, Ci (2A1) is in red, Hh is in green. Anterior is to the left.

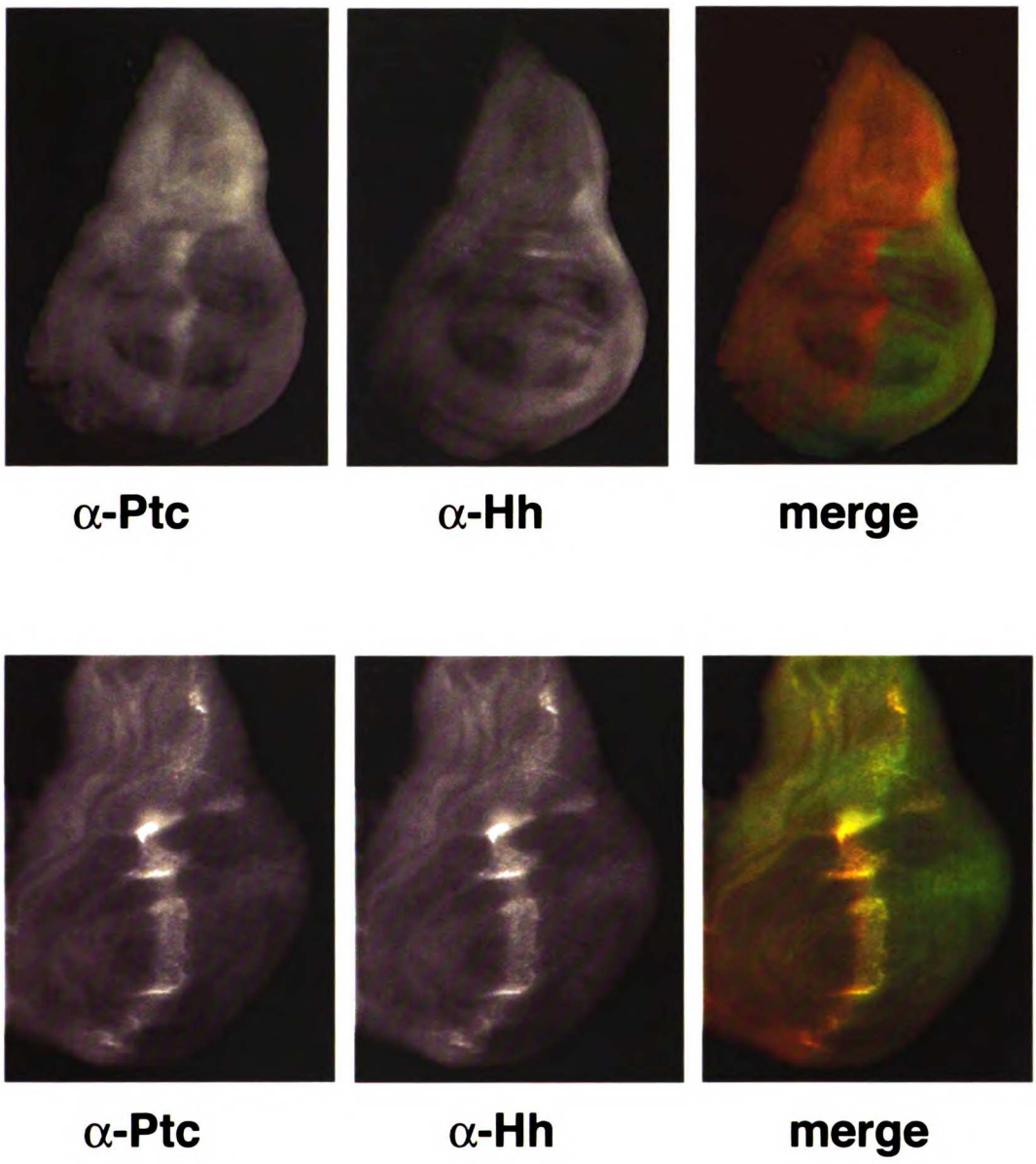


Figure 3. Hh and Ptc in discs expressing mutant Shibire
A) *ptc*-Gal4/+;UAS-*shi*^{ts}/+ discs that have been raised at the permissive temperateure. B) *ptc*-Gal4/+;UAS-*shi*^{ts}/+ discs that have been held at 32°C overnight. For both discs, Ci (2A1) is in red, Hh is in green. Anterior is to the left.

Effect of blocking endocytosis on signaling Blocking endocytosis in the anterior cells causes a change in the distribution of Hh, but it is not known whether there is an effect on signaling. To answer this question I looked at levels of Ptc and full length Ci in the Shi^{ts} expressing discs. Protein levels of both Ptc and Ci are dependent on activation of the Hh pathway; in response to Hh, Ptc transcript is highly upregulated, and full-length Ci is stabilized. The levels of Ptc did not change in the temperature-shifted *ptc*-Gal4; UAS-*shi*^{ts} discs (Figure 3). However, slow turnover rates make protein levels a poor readout for a reduction in signaling levels. Moreover, Ptc protein levels may be directly affected by the disruption of the endocytic pathway. Under normal conditions, staining with 2A1 reveals elevated levels of full-length Ci in anterior cells near the border (Fig. 2A). In temperature shifted *ptc*-Gal4; UAS-*shi*^{ts} discs, elevated staining sometimes appeared reduced (Fig. 2B), but this was not a reproducible phenotype. Although this data does not support a requirement for endocytosis in Hh signal transduction, more experiments will need to be done to rule out such a role.

Inactivation of Shi in the *ptc* domain leads to phenotypes in the adult wing. To gain further insight into the biological significance of Hh endocytosis, I examined adult wings of *ptc*-Gal4; UAS-*shi*^{ts} flies that had been shifted to the non-permissive temperature during development. Late 2nd and early 3rd instar larvae were shifted to 31°C overnight, then returned to room temperature and allowed to complete development. Results are summarized in Table 1. There was a high (> 60%) pupal lethality, and of escapers many (~20%) had no wings, or shriveled wings. I examined wings of those viable adults that had produced them, and found a slight, but highly penetrant, effect on the size of the intervein region between the 3rd and 4th veins (Fig. 5B). This is the region that is directly patterned by Hh, and this

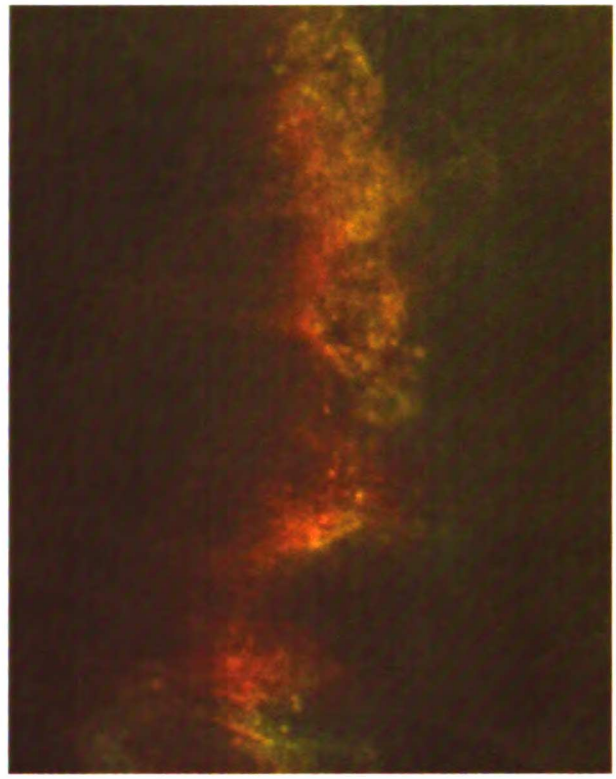


Figure 4. High magnification view of Hh accumulation in discs overexpressing Shi^{ts}
40x view of *ptc*-Gal4; UAS-*shi*^{ts} discs
Ptc is in red, Hh is in green. Anterior is to the left.

phenotype is similar to that of conditions in which there are lowered levels of Hh signaling, for instance weak mutations in *fu* (Therond, Limbourg Bouchon et al. 1999), or

Total pupae	Viable adults	Shriveled wings	Whole wings
~140	39	8	31

Table 1. Adult phenotypes of *ptc*-Gal4;UAS-*shi*^{ts} flies

overexpression of Ptc (Johnson, Grenier et al. 1995). The weakness of this phenotype is surprising, considering the high rate of lethality; however no intermediate phenotypes, such as fused veins, were apparent. Most likely, these widely variant phenotypes reflect a high sensitivity to the timing of the temperature shift.

The vein 3/4 phenotype could be due directly to a reduction in Hh signaling. However, it could also be a pleiotropic effect of the *shi* mutation, which affects all endocytosis in the cell. To address the question of specificity, I crossed the UAS-*shi*^{ts} flies to different drivers: *wg*-Gal4, which expresses at the dorsal-ventral margin, and *vg*-Gal4, which expresses throughout the disc early in development, but by the third instar is refined to the D-V boundary. The *wg*-Gal4; UAS-*shi*^{ts} flies had normal wings (Fig. 5 C), and there was no appreciable lethality. *vg*-Gal4; UAS-*shi*^{ts} flies were also largely normal, but in a small percentage there is a loss of tissue at the wing margin (Fig 5D). Neither driver gave a reproducible phenotype that was comparable to the effect of expression of Shi^{ts} in Hh responsive cells, suggesting that this effect might be the result of a specific repression of Hh signaling.

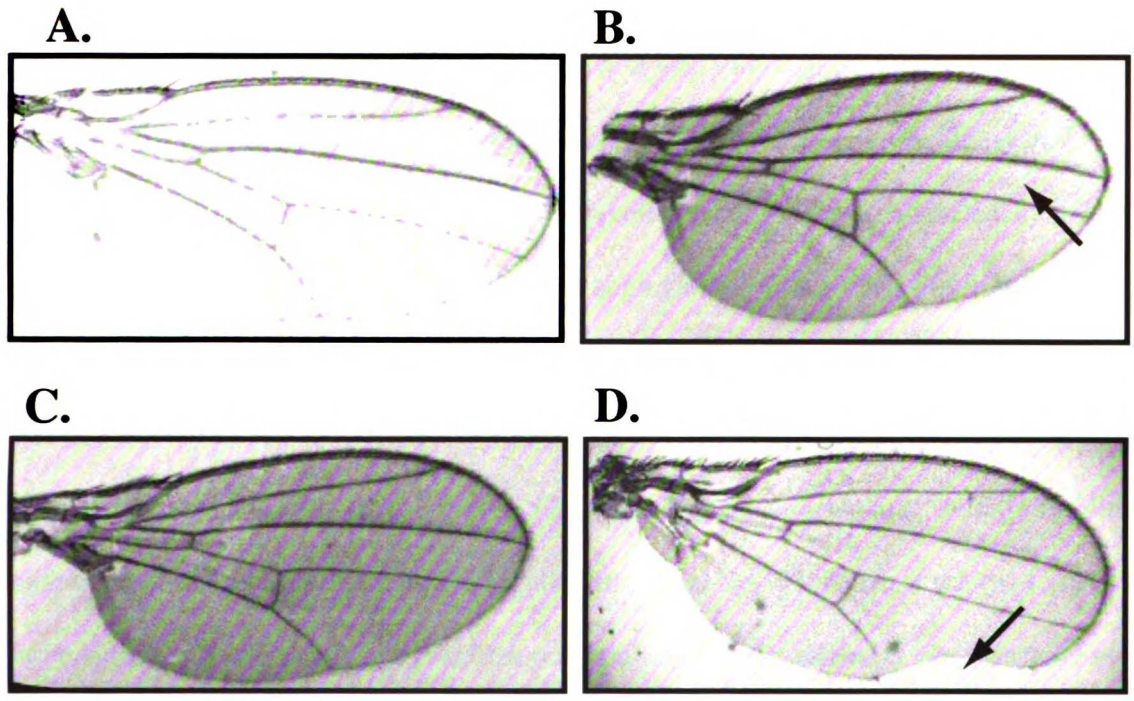


Figure 5. Phenotype of UAS-*shi*^{ts} expression on adult wings
A) *ptc*-Gal4 stock is generally wild type B) *ptc*-Gal4; UAS-*shi*^{ts}, note loss of tissue in intervein region (arrow) C) *wg*-Gal4; UAS-*shi*^{ts} D) *vg*-Gal4; UAS-*shi*^{ts}, wing margin defect is marked by an arrow.

Discussion

In this work I present evidence that dynamin-mediated endocytosis is involved in the sequestration of Hh in the imaginal disc. When endocytosis was blocked, Hh protein accumulated in the anterior compartment, where it was found co-localized with its receptor, Ptc. This observation supports a model in which Hh binds to Ptc and is endocytosed, followed by the targeting of Hh to a degratory pathway. Degradation could occur while Hh remains bound to Ptc, or could involve a later segregation of the two proteins with Hh being trafficked to late endosomes and lysosomes, and Ptc being recycled back to the surface. Recently two additional studies have come out also looking at endocytosis of Hh in imaginal discs. Torroja et al. (2004) and Han et al. (2004) both used clones of *shi*^{ts} cells in the anterior compartment to look at effects on Hh localization. Their results confirm many of my conclusions, as both groups observed Hh accumulation in the posterior compartment after shifting to the non-permissive temperature.

Examination of markers of Hh signaling in the imaginal disc provide little evidence that endocytosis affects Hh signaling in either a positive or negative fashion. In this study I found no reproducible effect of blocking endocytosis on levels of either Ptc or full-length Ci. Torroja et al. (2004) also looked at the effect of *shi* mutations on Hh signaling, using a *dpp*-lacz reporter, and also found no effect. However, it remains possible that there is an effect on signaling which was not observed using these markers.

The adult phenotype that results from inactivation of Shibire in the *ptc* domain is intriguing. The nature of the phenotype is similar to that of weak Hh pathway mutations, which together with the lack of a reproducible phenotype when Shi is temporarily inactivated in other regions of the disc might indicate a specific effect on the Hh pathway. However, this

is not supported by the molecular data in imaginal discs. This phenotype is unlikely to be a result of the high Hh levels that accumulate in this region, as an increase in Hh pathway activation is known to cause the opposite phenotype- an increase in the size of the vein 3/4 region. It is possible that looking in the adult wing provides a more sensitive assay, and allows us to see a subtle effect on Hh signaling that is not visible using larval markers. Alternatively, these cells may simply be more sensitive to the cell lethal effects of *shibire* mutations, causing cell death in this region, and hence a loss of tissue. A closer look at this phenomenon, for instance examining these discs with cell death markers, as well as with more sensitive reporters for Hh signaling in the disc, may shed light on the issue.

Materials and Methods

Fly stocks: Lab stocks used were:*ptc*-Gal4, *wg*-Gal4, *vg*-Gal4 and *hhlacZ*/TM3. The UAS-*shi*^{ts} was a generous gift of Gerold Schubiger. Wild type controls were performed with a *yw* strain.

Crosses: *ptc*-Gal4 x UAS-*shi*^{ts}, *wg*-Gal4 x UAS-*shi*^{ts}, *vg*-Gal4 x UAS-*shi*^{ts}

Immunostaining: Imaginal discs were fixed and stained as previously reported (Tabata and Kornberg 1994). Antibodies used were: rabbit α-Hh (Tabata and Kornberg 1994) at 1:10,000, affinity purified α-Ci mouse monoclonal 2A1 from Robert Holmgren (affinity purified by Brenda Ng) at 1:10,000, α-Ptc mouse monoclonal from Isabel Guerrero at 1:150.

Fly growth and heat shock: Flies were raised at room temperature (23-25°C) until the late 2nd to early 3rd instar larval stage. Temperature shifts were carried out at 31°C and incubated overnight. For imaginal disc stainings, late 3rd instar larvae were removed from non-permissive conditions, and dissected and fixed immediately. To assay adult wing

Chapter 4

Function of hydrophobic modifications of Hedgehog protein

Introduction

The experiments in this chapter address the role that hydrophobic modifications play in Hh movement and signaling. Evidence to date suggests that the major function of cholesterol modification is in regulation of Hh movement. Although both HhNp and HhN are able to signal, HhNp is more strongly membrane tethered in cultured cells (Porter, Ekker et al. 1996). However, as previously discussed, there has been conflicting evidence from flies and vertebrates as to the exact role of cholesterol modification in Hh distribution *in vivo*, with experiments in flies suggesting that HhN can travel further than modified Hh (Porter, Ekker et al. 1996), and experiments in the mouse limb bud showing the opposite (Lewis, Dunn et al. 2001). The importance of cholesterol in regulating Hh movement can be seen in the functions of the Disp transporter and Dlp, a heparin sulfate proteoglycan, both of which are required for proper distribution of full length HhNp, but not HhN (Burke, Nellen et al. 1999; Ma, Erkner et al. 2002; Han, Belenkaya et al. 2004).

Several interesting but poorly understood aspects of the Hh pathway also point to the link between Hh and cholesterol. Ptc, the putative Hh receptor, is a large protein that is predicted to have 12 membrane spanning helices. One of the domains of Ptc, encompassing several of these helices, has a high degree of homology to a conserved domain known as a sterol-sensing domain (SSD). SSD regions are found in a variety of proteins including several involved in cholesterol metabolism, the SREBP cleavage activating protein (SCAP), and

HMG CoA Reductase, as well as NPC-1, which is necessary for proper trafficking of cholesterol within cells (Carstea, Morris et al. 1997). All of these are membrane proteins that are able to respond to changes in cellular cholesterol levels. The homology of Ptc to NPC-1 is particularly high, extending throughout the protein sequence. A naturally occurring point mutation (D44A) in SCAP renders CHO cells insensitive to changes in cholesterol levels (Nohturfft, Hua et al. 1996). This residue is conserved throughout the SSD containing proteins, and when the corresponding mutation is made in Ptc it develops a dominant negative activity, inappropriately activating Hh target genes (Johnson, Zhou et al. 2002). However, this mutant Ptc protein seems to retain its ability to bind Hh, as it is able to sequester Hh when expressed in the posterior compartment (Johnson, Zhou et al. 2002). One additional phenotype of the Ptc SSD mutant is that it tends to accumulate in endosomes (Martin, Carrillo et al. 2001) This is interesting in light of the function of NPC, and may suggest that Ptc itself plays a role in vesicular trafficking, perhaps directing movement of Smo through endosomal compartments.

In addition, defects in cholesterol metabolism in mammals can give phenotypes similar to Hh pathway mutations (Cooper, Wassif et al. 2003), and compounds that mimic cholesterol structure, such as cyclopamine, have been shown to disrupt Hh signaling in vertebrates (Incardona and Roelink 2000). It was originally speculated that cyclopamine acts on Ptc, perhaps binding to its SSD, but its activity actually is through Smo (Taipale, Chen et al. 2000), suggesting that the links between the Hh pathway and sterols exist at many different levels (Taipale, Chen et al. 2000). These results, taken together with what has been learned from studies with of the Ptc SSD, leads to the interesting speculation that the activities of Ptc

and Smo can be regulated not only by their subcellular localization, but also by the biochemical properties of the membranes they reside in.

In addition to its cholesterol modification, mature Hh protein has a palmitoyl group attached to its N-terminal cysteine residue. While originally discovered in Sonic hedgehog, this modification also is present on fly Hh (Chamoun, Mann et al. 2001) and the function of this palmitoylation has been studied both in flies and in vertebrates. The vertebrate Hh has lower but still measurable activity when unpalmitoylated, as observed in cell culture assays (Pepinsky, Zeng et al. 1998). Mutants in the mouse acetyltransferase, Skinny hedgehog, show defects that are similar to loss of Hedgehog proteins, but they are less severe, indicating that not all activity has been lost (Chen, Li et al. 2004). In contrast, mutants in the *Drosophila* acetyltransferase show a complete loss of Hh signaling, and a point mutant of the N-terminal cysteine of Hh causes a dominant negative phenotype when expressed in otherwise wild-type animals (Chamoun, Mann et al. 2001). The reason for this discrepancy, as well as the mechanism by which palmitoylation increases signaling potency, is unknown.

In this chapter I present experiments that were designed to try to increase our understanding of the function of these two hydrophobic modifications in Hh signaling. First, I expressed proteins lacking one or both of these modifications in S2 cells, and compared their subcellular localization to that of fully processed Hh. I found that both hydrophobic groups are required for the membrane localization of Hh. Next, I expressed these Hh-FL and HhN proteins in anterior clones in the imaginal disc, and observed the subsequent induction of the target Ptc. There has been some confusion about the role of cholesterol in regulating the spread of Hh, due to the discrepancy between the vertebrate and fly results. Clonal analysis in the disc is a good system to address this question, as clones can be observed far

from the posterior source of Hh, allowing for specific analysis of the action of the protein being expressed. Finally, I delved into the question of the effect of the palmitoyl group on signaling. Again I used S2 cell expression, and observed the ability of modified and unmodified proteins to induce Fu phosphorylation in an autocrine manner. Using this highly sensitive autocrine assay, I found that fly Hh does retain some ability to signal when lacking its N-terminal palmitic acid, but not when it lacks both cholesterol and palmitoyl modifications. I also expressed this construct in flies, and confirmed the results of other groups that described a dominant negative effect when unpalmitoylated Hh is expressed in the wing.

Results

Hydrophobic modification alters membrane affinity of Hh. I expressed variant forms of Hh in S2 cells (shown in Chapter 1, Fig. 1D). In addition to the Hh-FL and HhN expressing constructs discussed in Chapter 2, I studied two different Hh proteins each with a point mutation in the N-terminal cysteine (C85). This mutation changes the palmitoylated residue, a cysteine, to serine, preventing this palmitoylation from occurring (Chamoun, Mann et al. 2001). Cysteine 85 is the most N-terminal residue left after cleavage of the secretory signal sequence, and when it was mutated to serine (C85S) the signal sequence was cleaved, but the serine was not palmitoylated. I used both a full length (HhCS) and an N-terminal (HhNCS) construct. Figure 1 shows the subcellular localization of these proteins. Hh-FL was localized at the plasma membrane, with some internal, vesicular staining (1A). In contrast, HhN was found mostly in cytoplasmic vesicles with little or no membrane staining (1B). These findings are consistent with the assumption that HhNp is more hydrophobic than HhN,

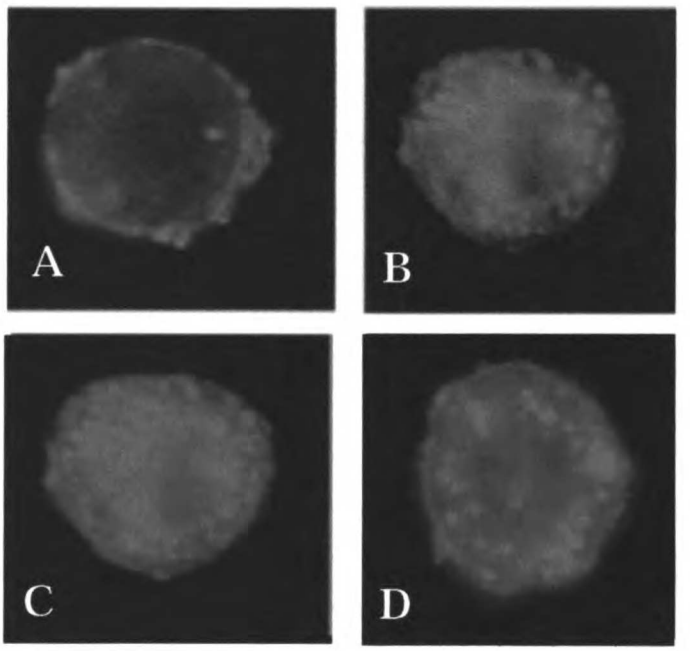


Figure 1. Localization different forms of Hh in S2 cells.
A) S2 cell expressing full-length Hh (visualized by anti-Hh Ab) note the strong staining in the plasma membrane.
B) S2 cell expressing HhN. Membrane staining is lost, and most of the protein is cytoplasmic.
C) S2 cell expressing full length cysteine mutant HhCS
D) S2 cell expressing cysteine mutant HhNCS

and that it is unlikely to diffuse freely away form cell membranes. Interestingly, ablation of palmitic acid modification had a similar effect. Expression of a Hh protein containing a mutation of the putative palmitoylation site resulted in loss of membrane localization, even when the C-terminal cholesterol was intact (Fig. 1 C and D). Hence, both hydrophobic modifications seem to be important for Hh localization.

Both full-length Hh and HhN are capable of signaling at a distance in flies. To examine the role of cholesterol modification for Hh movement *in vivo*, I made clones expressing Hh-FL and HhN in the anterior compartment of imaginal discs. Figure 2 shows clones in third instar discs stained for Hh and for Ptc, to mark the extent of target gene activation. Both the Hh-FL clones (Fig. 2A) and the HhN clones (Fig. 2B) induced Ptc expression outside of the clone (arrows). Furthermore, both discs had anterior compartments that had overgrown when compared to wild-type discs. There was variability in the extent of both Ptc expression and overgrowth. However, this variability depended on the timing and extent of clone induction, and did not correlate with expression of either form of Hh. Based on these results, I conclude that both molecules are capable of signaling across a distance of at least several cell diameters in *Drosophila*. More work will need to be done to determine with certainty whether HhN does have an increased range in the disc, as seen in the embryo. My data to this point does not support this conclusion.

Loss of the N-terminal palmitic acid reduces signaling by Hh. To examine the effect of palmitoylation on Hh signal transduction, I expressed the palmitoylation site mutants in S2 cells by transient transfection. After two days, cells were harvested and lysates were run on SDS-PAGE gels, and blotted for Fu protein. As can be seen in Figure 3, HhCS is capable of inducing formation of FuP, indicating an activated Hh signaling pathway. This is surprising,

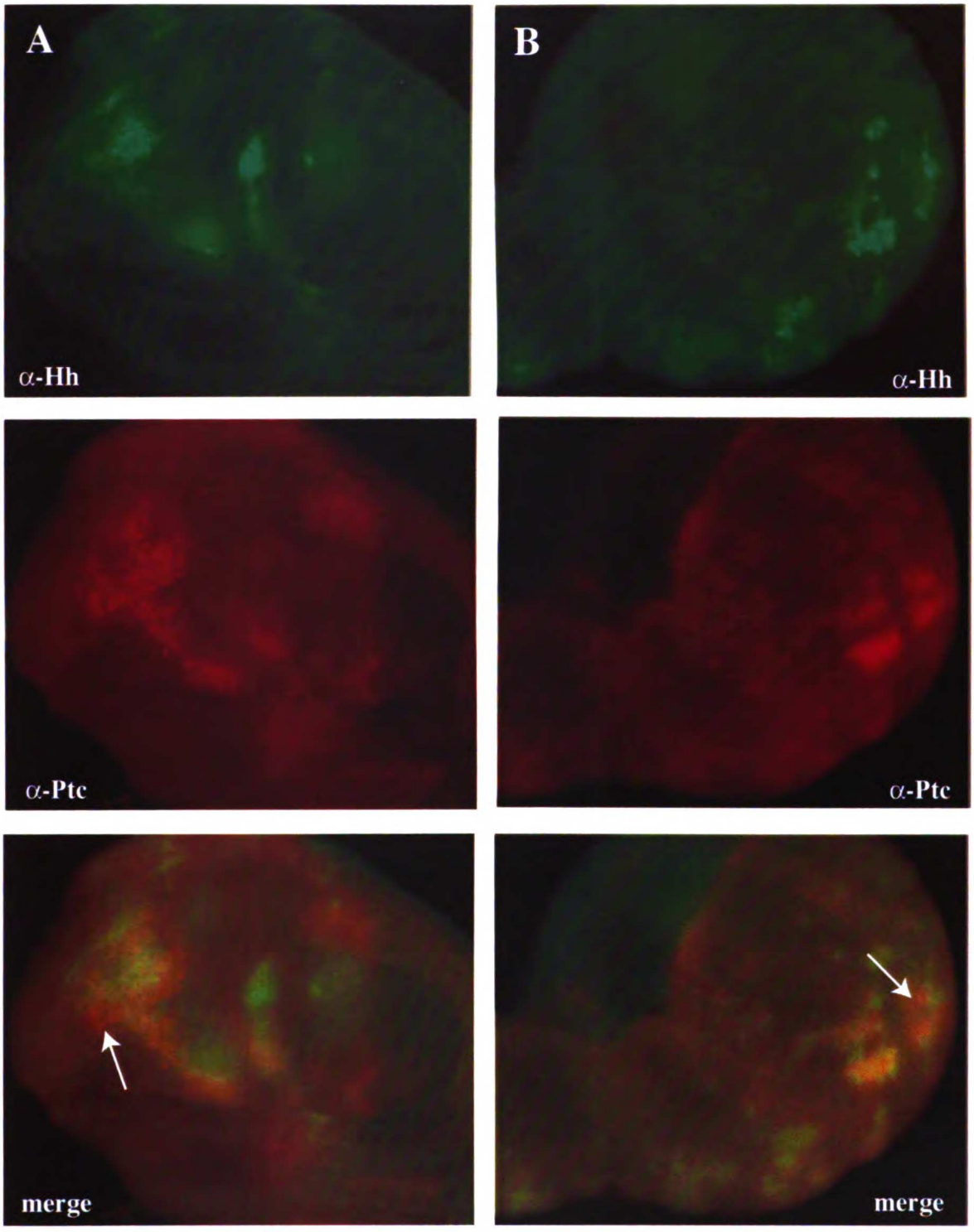


Figure 2. Clones expressing Hh in the imaginal disc
Clones were created with flp-Gal4 and stained with α-Hh (green) and α-Ptc (red). Arrows indicate areas of Ptc expression outside of Hh expressing clones A) UAS-Hh B)UAS-HhN

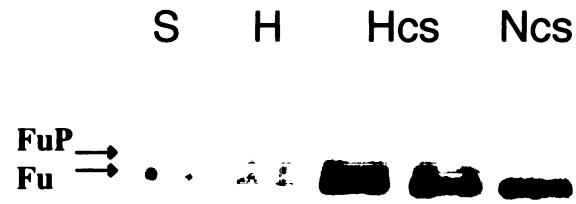


Figure 3. Effect of mutation of palmitylation site on Hh signaling.
Fu blot of cell lysates from cells expressing: no Hh (S), Hh-Fl (H), and the two palmitylation site mutants, HhCS (Hcs) and HhNCS (Ncs)

as previous work had indicated that unpalmitoylated fly Hh was not capable of signaling (Chamoun, Mann et al. 2001). This difference may be due to the increased sensitivity of assaying the signaling directly in the cells producing Hh. However, HhNCS, the form completely lacking hydrophobic modifications, is not active in this assay. This is not due to a lack of expression, as all transfected cells were tested for protein levels using either western blot or cell staining. According to my data, either the N-terminal palmitate or the C-terminal cholesterol modification by itself seems to be sufficient to activate signaling, but Hh with neither cholesterol nor palmitate is not. However, the underlying reasons for this requirement are unclear.

I created a transgenic *Drosophila* line to observe the effects of the HhCS mutant *in vivo*. Unfortunately, I was unable to obtain a transgenic line for the HhNCS construct. When expressed in the posterior under control of *engrailed*, the HhCS protein causes a dominant negative phenotype, shown in Figure 4C. Notice the loss of tissue between veins 3 and 4, and the fusions of these two veins, a common phenotype in conditions of reduced Hh signaling. A wild type wing and an *en*-Gal4/UAS-*ptc* wing, in which veins 3 and 4 are completely fused, are included for comparison. The dominant negative effect confirms the results of other labs (Chamoun, Mann et al. 2001). I was curious to determine whether this effect was due to competition for receptor binding in anterior cells, or to a disruption in secretion of Hh in posterior cells, perhaps by saturating the secretory machinery. To answer this question, I expressed the HhCS protein using the *en*103-Gal4 driver, which expresses at the extreme anterior end of the *en* domain, extending into the anterior compartment. As shown in Figure 4D, this produces the same dominant negative phenotype as expression in the broader *en*

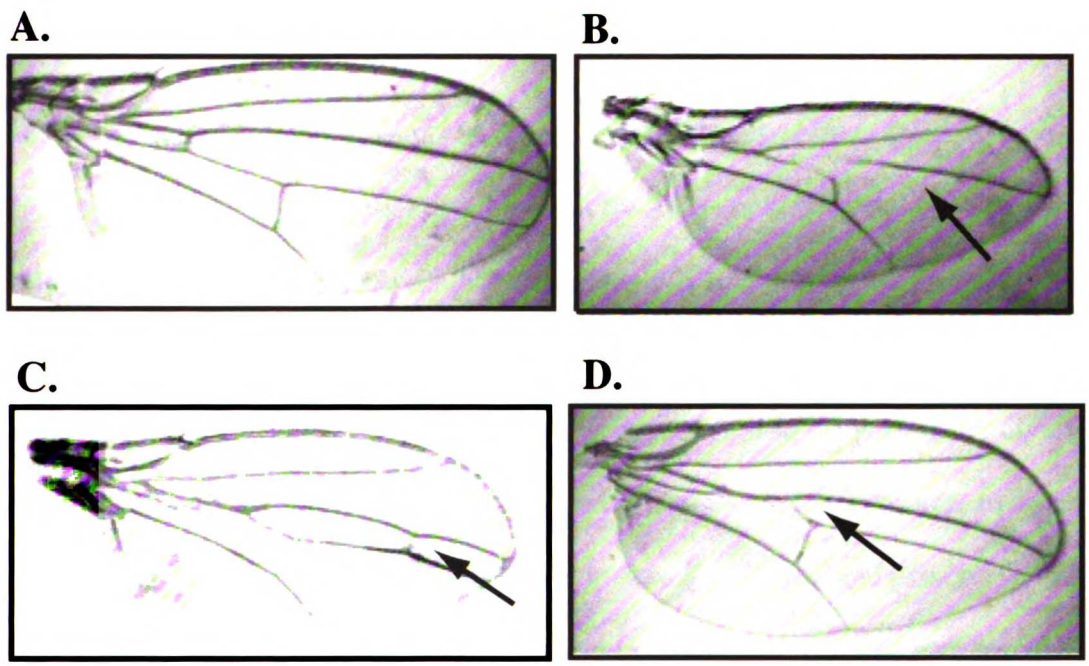


Figure 4. Phenotype of expression of palimitylation mutants on adult wings
A) Wild type wing, UAS-*hh*CS/+ B) In *en*-Gal4/UAS-*ptc* wings, veins 3 and 4 were fused. Both *en*-Gal4/UAS-*hh*CS and *en*103-Gal4/UAS-*hh*CS (C and D) wings exhibited a similar, although less severe, phenotype

domain, indicating that the effect of the mutant protein is most likely due to its action in the anterior compartment, where it may be interacting directly with Ptc.

Discussion

The two hydrophobic modifications of Hh have intrigued researchers since they were first discovered. Conflicting results, especially between fly and vertebrate systems, have clouded our analysis of the function of these modifications, but it is becoming clear that both cholesterol and palmitate are important for proper localization and signaling by Hh. Here, I show that both modifications are required for Hh to be localized to the plasma membrane of cultured cells. Despite this tight localization, the mature molecule *in vivo* is able to travel away from the cells that make it, as shown in previous work (Strigini and Cohen 1997; Zeng, Goetz et al. 2001), as well as in the clonal analysis presented here. A number of mechanisms exist to promote this movement, such as the promotion of secretion by Disp in the posterior (Burke, Nellen et al. 1999; Desbordes and Sanson 2003), and the activity of proteoglycans in the anterior (Desbordes and Sanson 2003). HhN, which lacks the cholesterol modification, may be able to bypass these mechanisms, as evidenced by is ability to move a distance away from the cells that make it, and its lack of dependence on Disp and Dlp.

The data from vertebrates, where ShhN has been claimed to be more restricted in its localization (Lewis, Dunn et al. 2001), complicates this model. It is possible that the difference between the two systems is due to the function of proteins such as Hedgehog interacting protein (Hip) a Hh binding protein that is present in vertebrates, but not in flies. Alternatively, it could be indicative of the difference between the distances involved. Free

diffusion of HhN may be sufficient to allow it to signal over the few cell diameters that are required in flies, while it is insufficient to produce a noticeable effect on Hh targets in vertebrates. In this case, the mechanisms that promote diffusion of HhNp would enable it to travel further than the diffusing HhN. Whatever the basis for the differences between HhNp and HhN, they do not seem to be the result of a major difference in their interactions with Ptc, as both are capable of normal signaling.

In contrast to the cholesterol modification, addition of a palmitoyl group to Hh seems to have its greatest effect on signaling potency. This was originally seen using Shh (Pepinsky, Zeng et al. 1998), and has also been shown in flies, where loss of palmitoyl modification has been previously reported to completely abrogate activity (Chamoun, Mann et al. 2001). While my results, using a sensitive autocrine assay, do not support the claim that unpalmitoylated Hh completely lacks signaling activity, I was able to confirm the dominant negative activity of this protein when expressed in flies. This suggests that the unpalmitoylated molecule is able to bind Ptc, but the signaling it promotes is not sufficient for normal development. The case of the completely unmodified molecule, HhNCS, is interesting. Using an assay for Fu phosphorylation, I show no activity for this protein. Unfortunately, I was unable to make a transgenic fly expressing this gene, but was reported at a recent meeting that HhNCS has no phenotype in the adult wing (K. Basler, meeting communication). If this is true, it may indicate that unmodified Hh is insufficiently targeted to the membrane to effectively bind Ptc. Palmitoylation may still play a role in Hh localization, as suggested by the alteration of the staining pattern in S2 cells. The reduced signaling activity of unpalmitoylated molecules makes it difficult to assay their movement *in*

vivo, but it is likely that loss of either hydrophobic modification will have an effect on the movement of Hh through tissue.

Materials and Methods

Cell culture: *Drosophila* S2 cells were cultured in Schneider media (Invitrogen), or in M3 media (Sigma), both supplemented by 10% FCS. All cell culture experiments were performed at 24°C. Transfections were performed using a Qiagen effectene kit, following the manufacturer's protocol. All proteins were expressed using the Gal4-UAS system (Brand and Perrimon 1993), by co-transfection of *actin*-Gal4 and a construct containing the gene of interest under control of the UAS promoter.

Immunohistochemistry: Imaginal discs were fixed and stained as previously reported (Tabata and Kornberg 1994). Cells were transferred to a glass chamber slide (Nalge Nunc), fixed in 4% formaldehyde in PBS, then stained with α-Hh and α-Ptc. All Hh stainings were performed at a dilution of 1:10,000. Ptc antibody was a mouse monoclonal from Isabel Guerrero, and was used at a dilution of 1:150.

Western blotting: All cells were lysed in NP-40 buffer (150mM NaCl, 10mM Sodium Phosphate buffer (pH 7.2), 1% NP40) plus DNase (50μg/ml) and RNase (25μg/ml). Blots were probed with rabbit α-Hh at 1:10,000 (Tabata and Kornberg 1994) or rabbit α-Fu at 1:10,000 (Therond, Alves et al. 1996), then with the secondary antibody, HRP α-rabbit (Jackson).

Fly stocks: Clones were made using a *yw* hs-flp; abx/Ubx<FRT f + FRT> Gal4-*LacZ* stock (de Celis and Bray 1997), crossed to either UAS-*hh*/CyO or UAS-*hhN*/CyO. Second instar larvae were heat shocked at 37°C for 15 minutes, then allowed to develop for one more day.

DNA constructs The HhCS and HhNCS expression constructs were made by inducing point mutants into the UAS-*hh* and UAS-*hhN* constructs, respectively, using the Quickchange kit from Stratagene.

Chapter 5

Discussion

During development, cells are thought to make fate decisions based on instructions from extracellular signaling molecules. These secreted molecules, called morphogens, disperse out from a source and produce a concentration gradient that can be interpreted by cells in a concentration dependent manner. The question of how such gradients arise in living tissues has been studied intensely. In my work, I looked specifically the Hh morphogen, an important protein in the development of most higher metazoans. This study, as well as the work of other labs, suggests that simple diffusion is an unlikely mechanism for Hh movement. Using a cell culture based system, I show that fully modified *Drosophila* Hh (HhNp) requires cell-cell contact for paracrine signaling. Although some HhNp can be found in the supernatant of Hh expressing cells, it does not appear to be active, nor is it taken up by naive cells. These findings lead to a model in which Hh normally signals in the context of direct membranes contacts between signaling cells.

The other major question that I address in this work is the function of hydrophobic modification in Hh movement and signaling. I found that both the cholesterol and palmitic acid modifications are required to tether Hh to the plasma membrane. However, my results suggest that each of these two modifications has a different function. Cholesterol modification seems to be primarily involved in controlling the movement of Hh. This may occur in two ways: by decreasing its ability to move in the extracellular environment, as previously seen in flies (Porter, Ekker et al. 1996), but possibly also by increasing its ability to be transported by active processes. Alternatively, cholesterol could be involved in the

formation of a soluble multimeric complex, as has been proposed for vertebrate Shh (Zeng, Goetz et al. 2001).

In contrast, the palmitoyl modification has a significant effect on the signaling activity of Hh. I show that, contrary to what has been reported (Chamoun, Mann et al. 2001), unpalmitoylated Hh retains some activity, but this activity is insufficient to fully induce target genes *in vivo*. Unfortunately, this deficiency makes it difficult to assay the effect of palmitic acid on Hh movement. Based on my finding that the palmitoylation defective mutant has a drastically altered localization in S2 cells, a hypothesis could be made that both the palmitoyl and cholesterol modifications contribute to restricting the free dispersion of Hh protein; however, this is still unproven. Also unclear is whether proteins involved in Hh secretion and transport, such as Disp and Dlp, interact differently with unpalmitoylated Hh, as is the case with HhN.

One interesting aspect of these two modifications is that they are not entirely independent. Addition of the palmitoyl group seems to be less efficient for Shh proteins lacking cholesterol modification (Taylor, Wen et al. 2001). This means that, when expressed *in vivo*, a percentage of HhN will be unpalmitoylated. Although the population of ShhN molecules which lacks cholesterol but retains the palmitoyl modification is as active as the dually modified form, ShhNp, *in vitro*, the *in vivo* potency of ShhN, and presumably *Drosophila* HhN, may be reduced if the majority is found in an unpalmitoylated state.

Hydrophobic modification endows Hh with a strong affinity for membranes. This has led to speculation that fully modified Hh is incapable of separating itself from its source without some unidentified active process. In the imaginal disc, Hh is produced by cells in the posterior compartment, and moves across the compartment border to signal to anterior cells.

It is known that Hh can signal directly to adjacent cells even when it is prevented from moving beyond the cells that make it. This was shown in the disc by experiments that expressed a membrane tethered form of Hh (Strigini and Cohen 1997), as well as by the formation of clones of anterior cells at the A/P border that cannot produce HSPGs (Takei, Ozawa et al. 2004). In both cases, Hh target genes were activated in the first row of cells adjacent to the Hh expression domain, which make direct contact with the Hh expressing cells. In contrast, cells anterior to this row did not show evidence of Hh signal transduction. Signaling in this first row of anterior cells was not sufficient to restore wild-type levels of Hh activity, indicating that Hh normally signals to cells a distance from the cells that produce it. However, the mechanism responsible for reception of the Hh signal by these more anterior cells has remained an open question.

My work presents evidence that free diffusion is not a likely mechanism for movement of Hh. This conclusion is supported by theoretical work which has argued that, due to trapping by their receptors, morphogens can not form the observed gradients by diffusion alone (Kerszberg and Wolpert 1998; Kruse, Pantazis et al. 2004). Also suggestive of an active process for Hh movement is the observation Hh cannot move into the anterior compartment on its own, but requires additional proteins, such as Disp and HSPGs.

However, the my data appears to conflict with work in vertebrates that shows paracrine signaling by a soluble protein complex containing modified ShhNp that was isolated from limb buds or conditioned media. This protein complex has a high molecular weight, and seems to represent a multimeric form of Hh, possibly containing other proteins and lipids as well. Furthermore, the complex has been proposed to have a micellar conformation, which

would allow it to increase its solubility by burying the cholesterol and palmitoyl groups inside a hydrophobic core.

While HhNp was found in Hh-FL conditioned media in my experiments, this protein was not active, suggesting that it does not correspond to the active, multimeric Shh seen in the vertebrate studies. It is possible fly Hh does form such a complex, and that this is the active signaling complex. However, the cells I used in this study, which were active in a paracrine signaling assay, either did not make the complex, or did not secrete it in sufficient concentrations to be active in my assays of the tissue culture conditioned media. It may be possible to generate cells that can produce higher concentrations of soluble Hh, but it is unclear if experiments performed with artificially high concentrations of Hh are relevant to the mechanism of signaling *in vivo*. If diffusion is a major factor in Hh distribution, it is most likely aided in its movement by the components of the complex itself, or by the actions of extracellular proteins such as the HSPGs, which are known to be required for Hh movement (Desbordes and Sanson 2003).

If Hh does not move by diffusion, how does it move? Various models have proposed: Hh could move from cell to cell through the endocytic pathway, a process called planar transcytosis. This has been proposed for other signaling molecules such as Dpp (Entchev, Schwabedissen et al. 2000). Hh could also pass directly from cell membrane to cell membrane, bypassing the endocytic pathway. Alternatively, cells away from the border could receive Hh directly using long cytoplasmic extensions, called cytonemes, which are known to be produced by these cells (Ramirez-Weber and Kornberg 1999).

In the first model, transcytosis, a morphogen would enter a cell through endocytosis, travel across the cell through the vesicular trafficking pathway, and be secreted by exocytic

machinery on the other side to be picked up by a neighboring cell. This model would not require diffusion, avoiding the possible problem of the morphogen interacting with membranes or extracellular proteins. However, in the case of Hh the planar transcytosis mechanism is not supported by my data, or by data that others have reported. As I show in Chapter 3, HhNp can pass into the anterior compartment in the absence of dynamin activity. Han et al (2004) and Torroja et al (2004) have reached similar conclusions. Indeed, Hh endocytosis seems more likely to be part of the mechanism that restricts its movement, through sequestration and targeting to the degratory pathway.

Although endocytosis does not seem to be required for Hh movement, Hh protein could still be passed directly from cell to cell. In this model of cell-mediated diffusion, hydrophobic Hh would diffuse along the apical surfaces of epithelial cells, in the plane of plasma membrane, possibly localized to lipid rafts (Rietveld, Neutz et al. 1999). Movement of Hh from cell to cell could be facilitated by Dlp or other glycoproteins, providing an alternative explanation for the requirement for these proteins in Hh movement. It has also been suggested that morphogen receptors could play a role in this type of movement, by trafficking morphogens across membranes, or by passing the proteins from cell to cell (Kerszberg and Wolpert 1998). Such an activity by Ptc cannot be a requirement for Hh movement, as Hh can pass through tissue that lacks Ptc (Chen and Struhl 1996). However, it is possible that Ptc in the wild type situation is capable of aiding Hh movement from cell to cell, or that an as yet unknown Hh-binding molecule performs this function. One potential caveat of this mechanism is that it is uncertain if it would be able to function in mesenchymal tissues where Hh is known to signal, such as the interior of the vertebrate limb bud.

Cytonemes are long, actin-based filopodia produced by cells of the imaginal disc. Intriguingly, they seem to be polarized, with cells at a distance from the compartment border producing cytonemes that extend towards the border, a source of developmental signals. This led to the proposal that they are involved in the reception of morphogens, particularly Hh and Dpp (Ramirez-Weber and Kornberg 1999), although how they would accomplish this is unknown. One idea is that they could have receptors localized to their tips, thus allowing direct reception of Hh signal. Supporting this idea, a GFP fusion of Thickveins (Tkv), the Dpp receptor, has been seen in punctate structures that could correspond to cytoneme tips (Ramirez-Weber and Hsiung, personal communication). Alternatively, Hh or other morphogens could use these membranous filaments to travel back to the cell bodies of receiving cells. The requirement for HSPGs would be explained in this model if they were required as a substrate for cytoneme growth or for stabilization of cytonemes.

If cytonemes are responsible for morphogen signaling at a distance, a question that must be addressed is how cells are capable of responding in a graded fashion. If each cell could reach to the source of morphogen, they should all receive the same level of signal, in which case there would be no activity gradient. This problem could be solved if cells were capable not only of receiving the signal, but of determining their distance from it. If reception occurs at the tips of cytonemes, the response of the signal transduction pathway itself could be graded, with shorter cytonemes allowing a stronger signal to reach the nucleus. Alternatively, if the morphogen itself is able to travel along cytonemes, the gradient could form in the membrane of the cytoneme itself. In this case, receptors on the cell surface would see a higher concentration of morphogen as a function of proximity to the source. Another model is that cytonemes can respond to the surrounding environment, forming multiple stable

contacts, depending on their length and on the strength of the signal. This model fits well with the observations that cytoneme growth is highly dynamic, and that they are capable of forming complicated, branched structures (Ramirez-Weber and Kornberg 1999).

Hh movement across membranes and movement along cytonemes are equally valid models at this time. Hopefully, in the future our ability to manipulate filopodia formation and membrane properties *in vivo* will improve, and will allow experiments to help distinguish between the two models. In addition, development of a single cell assay for Hh signaling, which would allow Hh movement and signaling to be followed visually in individual cells, would greatly benefit our understanding of this process.

My work here, as well as that of many other labs in recent years, shows that the movement of Hh is a complex process with many different mechanisms involved, both promoting and restricting spread of the protein. A similar picture is arising of the formation of gradients of other morphogens. The mechanisms of movement of both Wg and Dpp have been intensely studied. The transport of Wg has many aspects which appear to be similar to that of Hh. Movement of Wg has been shown to require proteoglycans (Tsuda, Kamimura et al. 1999), and hydrophobic modification of Wg has also been observed (Zhai, Chaturvedi et al. 2004). Transcytosis has also been proposed as a mechanism for Wg movement, but experiments using *shi*^{ts}, similar to those performed for Hh, give evidence that this is not the case (Strigini and Cohen 2000). A unique mechanism that has been suggested for Wg transport is that it is carried by proliferating cells in the *Drosophila* embryo (Pfeiffer, Ricardo et al. 2002). In this model, cells that produce Wg would move away from the parasegment boundary, and secrete Wg protein, allowing cells in their new environment to receive the signal. This may be a mechanism for Wg movement in the embryo, but the larger distances

and slow rate of proliferation in the disc make it unlikely to be important there (Vincent and Dubois 2002). The evidence against active mechanisms of Wg transport have led to the conclusion by researchers that Wg moves by free diffusion, but there is no evidence that such diffusion actually occurs *in vivo*.

The case of Dpp is even more complicated, with many conflicting reports. Particularly, the question of whether transcytosis is involved in Dpp movement has been debated, with some groups reporting that endocytosis is required (Entchev, Schwabedissen et al. 2000; Kruse, Pantazis et al. 2004), and others presenting evidence of an endocytosis independent mechanism (Belenkaya, Han et al. 2004). When clones of *shi*^{ts} cells were made in the disc, shadows of reduced Dpp levels were noted behind these clones, leading to the conclusion that Dpp cannot move through tissue defective for endocytosis (Entchev, Schwabedissen et al. 2000). However, it has been proposed that this effect is actually due to an increase in levels of Tkv within the clones, which may retard the spreading of Dpp, in a manner similar to the action of Ptc (Lander, Nie et al. 2002). Furthermore, levels of phosphorylated Mothers against Dpp (p-MAD), a marker for activation of the Dpp pathway, were not reduced behind the *shi*^{ts} clones, indicating that sufficient Dpp can pass through the dynamin deficient cells to signal (Belenkaya, Han et al. 2004). Mathematical modeling has also been employed to explore this problem, but the conclusions of these models differ depending on the initial assumptions made (Lander, Nie et al. 2002; Kruse, Pantazis et al. 2004).

Attempts have also been made to directly image the distribution of Dpp protein. When the localization of a GFP-Dpp fusion protein was observed *in vivo*, it was found in an intracellular gradient, in punctate structures that are presumed to be endocytic (Teleman and Cohen 2000). However, the majority of GFP-Dpp in the wing disc was accessible to

proteinase K, implying that it was extracellular. At the same time another group, using a the same GFP-Dpp fusion, but employing a technique that purports to allow specific staining of extracellular protein, detected only a weak gradient of extracellular GFP staining (Entchev, Schwabedissen et al. 2000). More recently, a study that used the same protocol but a different α–GFP antibody, found extracellular localization in a broad gradient that more closely matched the observed distribution of p-MAD (Belenkaya, Han et al. 2004; Kruse, Pantazis et al. 2004). There have also been reports that a portion of the extracellular Dpp pool is inside the lumen of the disc, between the disc proper and the peripodial membrane (Gibson, Lehman et al. 2002).

This conflicting data could be explained by a mechanism that involves both free diffusion and transcytosis, with Dpp able to diffuse for short distances through Shi^{ts} clones, but normally requiring transcytosis for long-range movement (Kruse, Pantazis et al. 2004). Alternatively, Dpp could move by neither mechanism, but instead employ one of the cell-based mechanisms that have been suggested to describe Hh movement.

Another open question is to what degree mechanisms of movement are conserved between morphogens. These proteins perform similar roles in development, and it may be that they utilize similar transport mechanisms. It is already known that many aspects of their movement, such as dependence on proteoglycans, have been conserved. However, there are certain properties of movement that differ between morphogens. Hh and Dpp, for example, have vastly different ranges of signaling in the wing disc. Also unclear is the degree to which mechanisms are conserved between species. I have already discussed some of the apparent differences that have been observed in Hh movement in vertebrates and flies. Vertebrate systems may present new challenges for morphogen movement, because of the greater

distances involved, as well as the need for proteins to move through non-epithelial tissue. To this date the genetic tools available in the fly imaginal disc have allowed detailed analysis of morphogen movement in this system. Unfortunately, analysis in other species has lagged behind.

Although much work still needs to be done to determine the exact mechanisms, the original idea of morphogen gradient formation as a passive process is disappearing, replaced by models of active processes. Whether involving endocytosis, transport by extracellular proteins, or filopodial extensions, gradient formation is a dynamic event, which is highly regulated by the tissues it occurs in. This regulation may be necessary to create the proper gradient shape for correct cell fate determination. In addition, it may allow the system to respond to perturbations while maintaining a consistent developmental program. For instance, an increase in production of the morphogen could be counteracted by clearing excess protein through endocytosis. Overall, this new picture of morphogen gradients enhances our understanding of the process of development.

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