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How does cholesterol affect the way Hedgehog works?

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Members of the Hedgehog (Hh) family of proteins are conserved morphogens that spread and modulate cell fates in target tissue. Mature Hh carries two lipid adducts, a palmitoyl group at the N terminus and cholesterol at the C terminus. Recent findings have addressed how these lipid modifications affect the function and transport of Hh in *Drosophila*. In contrast to the palmitoyl adduct, cholesterol appears not to be essential for signalling. However, the absence of the cholesterol adduct affects the spread of Hh within tissues. As we discuss here, the exact nature of this effect is controversial.

Introduction

A relatively small number of secreted proteins spread through tissues during development and organise tissue patterning. Understandably, there has been great interest in the mechanisms that modulate the range of action of these developmental ligands. So far, three such secreted developmental signals, Wingless/Wnt, Spitz (*Drosophila* Transforming Growth Factor α) and Hedgehog (Hh), are known to undergo lipid modification (Jeong and McMahon, 2002; Miura et al., 2006; Pepinsky et al., 1998; Porter et al., 1996; Willert et al., 2003). All three are palmitoylated in the lumen of the endoplasmic reticulum (ER) during biosynthesis. In addition, Hh also undergoes cholesterol modification (Porter et al., 1996). Although the palmitoylation of cytoplasmic proteins has been extensively studied, the palmitoylation of proteins residing in the lumen of ER-derived vesicles has only been recently recognised, and the enzymology of these reactions is still poorly understood. In the cases of Spitz and Hh, the reaction appears to be catalysed by the product of the gene *skinny hedgehog*, which is also known as *rasp* or *sightless* (Amanai and Jiang, 2001; Chamoun et al., 2001; Lee and Treisman, 2001; Micchelli et al., 2002). By contrast, more work is needed to confirm that Wingless palmitoylation is catalysed by the gene product of *porcupine*, as has been suggested (Kadowaki et al., 1996; Willert et al., 2003).

Interest in the role of lipid adducts in morphogen signalling and activity is heightened in the case of Hh because it undergoes a second lipid modification. The N-terminal half of Hh becomes conjugated with cholesterol by an autocatalytic reaction that simultaneously cleaves off the inactive C-terminal polypeptide (Porter et al., 1996). Thus, mature active Hh, which is about half the size of full-length Hh, carries a palmitate group at the N terminus and a cholesterol moiety at the C terminus. After biosynthesis, Hh must be released from secreting cells. This requires the activity of a dedicated multipass-membrane protein encoded by the *dispatched* (*disp*) gene (Burke et al., 1999). There is still some controversy as to the exact mode of Disp action. One view is that, by virtue of its sterol-sensing domain, Disp allows the release of lipidated Hh from the cell surface (Burke et al., 1999). Alternatively, Disp has been suggested to guide Hh from the basolateral membrane to the apical membrane of the cell, where the release of Hh would take place (Gallet et al., 2003), thus allowing its subsequent extracellular transport.

Lipid modification is believed to cause proteins to tightly associate with cell membranes (Linder and Deschenes, 2004). Such an association is expected to cause lipidated ligands to remain stuck at the surface of secreting cells, thus preventing their subsequent transport. However, the transport of lipidated molecules, such as Hh, does take place. So, how is membrane association compatible with long-range transport and signalling? It has been suggested that specific transport mechanisms have evolved to overcome this problem. For example, lipid particles called argosomes have been proposed to extract lipidated ligands from the cell surface and then to act as vehicles for long-range transport (Eaton, 2006). According to this view, cholesterol would be required for transport, as it would be needed for the loading of a lipidated protein onto the transport vehicles.

Here, we review recent findings that address how lipid modifications of the highly conserved ligand Hh affect its signalling activity and range in *Drosophila* epithelia. One report (Gallet et al., 2006) suggests that cholesterol is indeed required for long-range Hh transport. Two other reports (Callejo et al., 2006; Dawber et al., 2005) propose that, in the absence of a cholesterol adduct, the spread of Hh is increased, although this is at the expense of its signalling activity near its source, perhaps because of the dilution of unlipidated Hh in the extracellular space. Below, we evaluate the evidence and then briefly relate it to the situation in vertebrate embryos.

Tools and tissues for the study of *Drosophila* Hh

The wing imaginal disc system

One system of choice to study the range of extracellular ligands is the wing imaginal disc of *Drosophila*. This structure, found in the growing larva, consists of an epithelial sack that has a thick, columnar, pseudostratified epithelium on one side and a squamous epithelium, called the peripodial membrane, on the other (Fig. 1B). The space between the two epithelia is called the peripodial space. It is the columnar epithelium in the central region of the disc (the pouch) that gives rise to the wing proper, hence most attention has been devoted to this tissue. Somewhat unusually for epithelia, the apical side of this tissue faces the inside of the disc (the peripodial space) (Pallavi and Shashidhara, 2005) (Fig. 1B). Hh is produced from the posterior half of the disc [the posterior (P) compartment], from where it spreads into the anterior (A) compartment (Basler and Struhl, 1994; Tabata and Kornberg, 1994) (Fig. 1A, parts a,b). It forms a gradient (over about 10 cell diameters) and activates a series of target genes in a concentration-dependent manner (Fig. 1A, parts c,d). Examples of 'high level' targets include *engrailed* (*en*) and *patched* (*ptc*), while *decapentaplegic* (*dpp*), *cubitus interruptus* (*ci*), *collier* (*col*) and *iroquois* (*iro*) are low level targets (Torroja et al., 2005). How the range of Hh is regulated so that it activates its targets in the right pattern is an issue of considerable interest.

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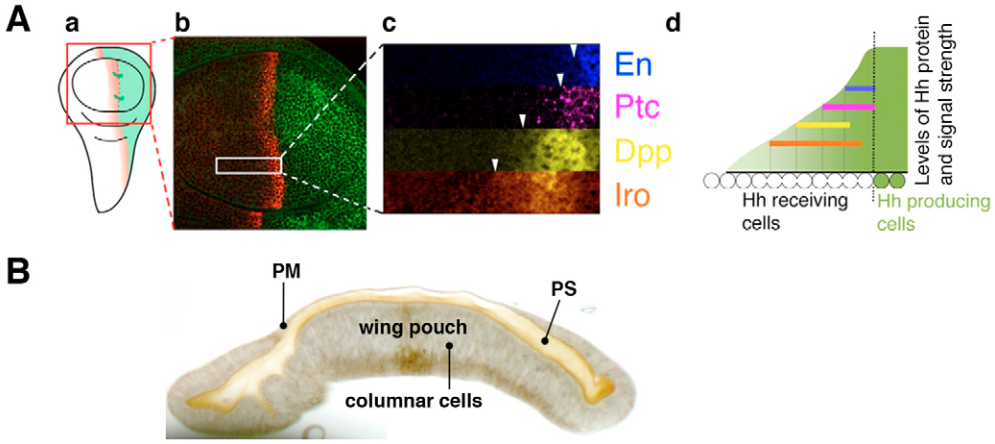

Fig. 1. Range of Hh activation in the wing disc. (**A**, part a) A schematic of *Drosophila* wing imaginal disc staining [adapted from Callejo et al. (Callejo et al, 2006)]. Anterior (A) is to the left. (a,b) Hh-producing cells in the posterior (P) compartment are shown in green. Ptc (red) appears as a stripe that abuts the AP boundary. (A, part c) Expression of relevant target genes. Engrailed (En, blue) and Patched (Ptc, pink) are high threshold targets. Decapentaplegic (Dpp, yellow) and Collier (not shown) are medium threshold targets, and Iroquois (Iro, orange) is a low threshold target. (A, part d) A graphic representation of target activation represented by the colours above, as a function of local Hh concentration. The vertical line represents the AP compartment boundary. (**B**) Cross-section of a wing imaginal disc. The wing pouch is covered by an epithelium called the peripodial membrane (PM). The lumen between the disc proper cells and the PM is the peripodial space (PS). Here, the PM is highlighted with horse radish peroxidase (HRP) (micrograph courtesy of Laurence Dubois, Centre de Biologie du Développement, Toulouse, France).

Modulators of Hh distribution

Several proteins that are expressed in Hh-receiving tissues have been shown to affect the range and distribution of Hh after its release from secreting cells. One class of such proteins comprises the Heparan Sulfate Proteoglycans (HSPGs) *dally* and *dally-like* (*dlp*) (Bellaiche et al., 1998; Bornemann et al., 2004; Han et al., 2004a; Han et al., 2004b; Takei et al., 2004; Takeo et al., 2005). Dally-like is required for Hh signalling itself, perhaps by acting as a Hh co-receptor (Desbordes and Sanson, 2003; Han et al., 2004b; Lum et al., 2003). *Dlp* and *dally* might also participate in transport, although a cell biological understanding of how they achieve this is not yet available (Desbordes and Sanson, 2003; Glise et al., 2005; Gorfinkiel et al., 2005; Han et al., 2004b; Lum et al., 2003). Another protein that might be involved in the distribution of Hh is the secreted molecule encoded by *shifted*. Shifted, which is related to vertebrate Wnt inhibitory factors, such as WIF-1 (Hsieh et al., 1999), can diffuse readily within imaginal discs (Glise et al., 2005; Gorfinkiel et al., 2005). Genetic data have suggested that it could mediate the interaction between Hedgehog and HSPGs (Glise et al., 2005; Gorfinkiel et al., 2005). Despite its homology to WIF-1, Shifted does not modulate Wingless activity in flies (Gorfinkiel et al., 2005). Another important molecule that modulates the range of Hh is Patched (Ptc). This multipass-transmembrane protein, which is the primary receptor for Hh, represses downstream signalling in the absence of Hh. Upon Hh binding, this inhibition is relieved and downstream Hh targets are activated. The *ptc* gene itself is a target of Hh signalling, and high level expression of Ptc sequesters Hh by endocytosis, thereby limiting its further transport (Chen and Struhl, 1996; Chen and Struhl, 1998; Torroja et al., 2004). Thus an increased production of Hh leads to more of it being sequestered. This negative-feedback loop ensures that the range of Hh is always kept under control.

Experimental variants of *Drosophila* Hh

Three groups have sought to assess how lipid modifications affect the range of Hh by expressing mutated forms of Hh that are expected to lack either or both lipid adducts (Callejo et al., 2006; Dawber et al., 2005; Gallet et al., 2006). The various forms of Hh used were expressed in flies using the Gal4/UAS system, which allows the expression of so-called UAS transgenes in specific patterns (Brand and Perrimon, 1993). Although extremely powerful, this system has the drawback that the transgenes are usually expressed at a substantially higher level than are endogenous genes. Several forms of Hh were expressed in this fashion: $Hhp^{palm;choles}$, HhN^{palm} and $HhC85S^{choles}$, as discussed below. For ease, we use a unified nomenclature to describe the various constructs used in these experiments, as the key mutations were identical in all cases, even though they were made independently in the three laboratories. The constructs differed in that some were tagged with GFP while others were not. However, extensive evidence suggests that the GFP tag does not affect the signalling or trafficking behaviour of Hh (Callejo et al., 2006; Torroja et al., 2005; Gorfinkiel et al., 2005; Burke et al., 1999; Dawber et al., 2005). The production of $Hhp^{palm;choles}$ results from expression of the full-length form of Hh, which is processed during its biosynthesis to give rise to mature active Hh (the N terminal-half of pro-Hh, bearing a lipid adduct at each end; Fig. 2). HhN^{palm} represents the N-terminal half of pro-Hh without the cholesterol moiety (Fig. 2B). In $HhC85S^{choles}$, the cysteine at position 85, which is the target of palmitoylation in wild-type Hh, is mutated to a serine (Fig. 2C). This form of Hh is strongly impaired in signalling (both in vertebrates and *Drosophila*), implying an essential role for the palmitoyl group in signal transduction (Callejo et al., 2006; Dawber et al., 2005; Gallet et al., 2003; Pepinsky et al., 1998). By contrast, HhN^{palm} is signalling competent. It is assumed that the lack of cholesterol does not preclude palmitoylation, otherwise it would also be signalling incompetent (Burke et al., 1999; Chamoun et al., 2001). Because target gene expression provides an important readout of the range of Hh signalling, we will not consider the palmitate-deficient forms of Hh any longer and will focus on the effect of cholesterol on the range of Hh in *Drosophila*.

The activities of cholesterol-deficient Hedgehog

Cholesterol and Hh multimerisation

As expected, cholesterol mediates the association of mature Hh with the plasma membrane. This was shown in both *Drosophila* S2 cells and mammalian cells (HEK293T) (Chamoun et al., 2001; Chen et al., 2004; Gallet et al., 2006; Zeng et al., 2001). Cells expressing $Hhp^{palm;choles}$ showed a strong accumulation of Hh at the cell surface, whereas cells expressing HhN^{palm} at a comparable level did not. Fractionation studies of the supernatant of Hh-expressing cells

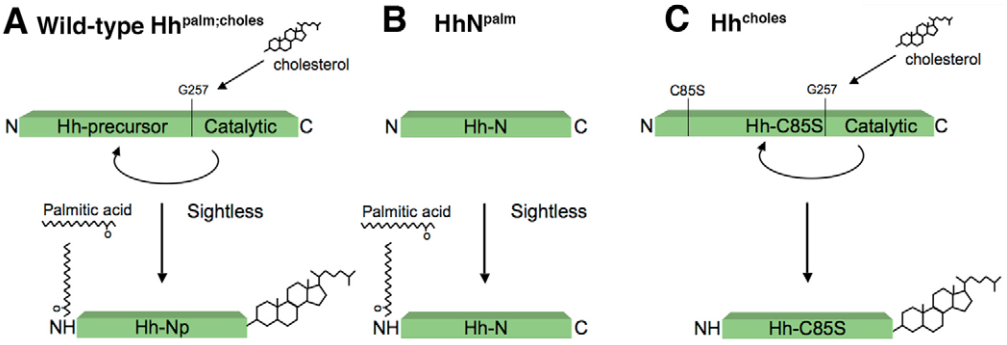

Fig. 2. Hh processing and activity in *Drosophila* wing imaginal discs. (**A**) A schematic of Hh processing, which involves the removal of the C-terminal half at G257 and the concomitant addition of cholesterol. Palmitic acid is also added onto a N-terminal cysteine, in a reaction catalyzed by Sightless, a membrane bound O-acyltransferase. (**B**) HhN^{palm} consists of only the N-terminal part of Hh, which is not cholesterol modified, as the catalytic C-terminal part is missing. The addition of palmitic acid to this form is thought to be unaffected. (**C**) $HhC85S^{choles}$ contains a point mutation, replacing cysteine 85 with serine. This form of Hh carries cholesterol but no palmitoyl adduct.

showed that $Hhp^{palm;choles}$ participates in high-molecular weight structures that are likely to represent mutimeric complexes (Callejo et al., 2006; Chen et al., 2004; Gallet et al., 2006; Zeng et al., 2001). HhN^{palm} is not found in these structures, suggesting that cholesterol mediates multimerisation, as has been shown before for vertebrate Shh (Chen et al., 2004; Goetz et al., 2006; Zeng et al., 2001). Gallet et al. attempted to assess the activity of the different fractions obtained by size exclusion chromatography from the cell culture supernatant. Although it is inherently difficult to ensure that comparable amounts of Hedgehog are used for each fraction, the authors provided good evidence that the multimeric fraction is more active than the monomeric one. They concluded from these results that cholesterol modification (and multimer formation) ensures high signalling activity. Because the monomeric fraction retains some signalling activity, it appears that cholesterol is not essential for signalling although it may contribute to its maximal activity.

Subcellular localisation of Hh

Gallet et al. found that wild-type Hh is mostly present on the apical side of the columnar cells in *Drosophila* imaginal discs (Gallet et al., 2006). From this observation, they suggest that, following biosynthesis, wild-type Hh is released apically and then transported along the apical side of the columnar epithelium. The cholesterol adduct could be important for this as they found that (untagged) HhN^{palm} accumulates at a basal localisation. Callejo et al. also believe that wild-type Hh is secreted apically (Callejo et al., 2006). However, they suggest that it then finds its way to the basolateral domain, where it is transported to and then activates downstream signalling. They too stained fixed imaginal discs, although they followed a staining protocol that is designed to detect specifically extracellular GFP-tagged forms of Hh produced from clones of cells. This was achieved by inducing, at random locations, clones of cells that activate the expression of *gal4*. Gal4 in turn activates the relevant forms of Hh (this is colloquially called the 'Flp-Out' technique) (Harrison and Perrimon, 1993; Struhl and Basler, 1993). Callejo et al. found that, at the surface of Hh-producing cells, all forms of Hh are found mainly at the apical surface, consistent with apical secretion. In the receiving cells, wild-type Hh is basolateral, whereas HhN^{palm} remains at the apical surface, as if cholesterol was needed for the transfer of Hh from one side to the other. In order to map where, along the apicobasal axis, various Hh variants are internalised, they looked at Hh variant distribution following a transient block of endocytosis. Consistent with their reported steady-state distribution, they found that wild-type Hh accumulates basolaterally in receiving cells, while HhN^{palm} seems to build up at an apical location. Overall, Callejo et al. argue that cholesterol-modified $Hhp^{palm;choles}$ diffuses along the basolateral surface, while unmodified HhN^{palm} spreads apically.

Here, it is relevant to note that the HSPG *dally-like*, which contributes to Hh transport (Han et al., 2004a; Han et al., 2004b; Takei et al., 2004), is relatively more abundant at the basolateral surface of the columnar epithelium (Kreuger et al., 2004; Marois et al., 2006). The differences between the observed distributions of Hh variants may be due to the differences between the assays. In one case (Gallet et al., 2006), the steady-state distribution of total (intra- and extracellular) endogenous Hh was measured, whereas, in the other (Callejo et al., 2006), the extracellular distribution of overexpressed ligand was assessed. Clearly, total staining does not necessarily give an indication of the routes of export/transport, nor does it tell us whether the ligands are inside or outside of the cells. Extracellular staining is more specific, but it can give rise to artefacts that are caused by a molecule's limited access to the apical side of a wing disc cell (as this side is inside the disc). In addition to looking at extracellular steady-state distribution, Callejo et al. also stained discs following a block of endocytosis. The results of this experiment seem to confirm that wild-type Hh is mainly present at the basolateral side in receiving cells, although it should be kept in mind that a block of endocytosis will necessarily affect the distribution of a large number of cell surface molecules, some of which might be needed for Hh transport. Overall, we believe that novel methods to assess the trafficking of Hh in imaginal discs are needed to resolve the divergent views on the nature of the epithelial surface where Hh spreads and signals. Better techniques will also be needed to assess the distribution of the relevant Hh receptors. Presumably, they are present on the epithelial surface where transport and signalling are taking place. The HSPG co-receptor Dlp is thought to be mainly, although not exclusively, present at the basolateral surface (Kreuger et al., 2004; Marois et al., 2006), whereas Ptc has been reported to be mainly apical (Capdevilla et al., 1994; Gallet et al., 2005). This also needs confirmation.

Does cholesterol reduce or extend the range of Hh?

A simple assay that measures the range of Hh transport and signalling involves inducing the expression of different forms of Hh in groups of cells (using the Flp-Out technique) and monitoring the ensuing protein distribution and activation of target genes. In one set

of experiments, Gallet et al. found that, in comparison to $Hhp^{palm;choles}$, HhN^{palm} activates the target gene *ptc* and the target reporter *dpp-lacZ* over a relatively short range (Gallet et al., 2006). From these data, and from additional results obtained in the *Drosophila* embryonic epidermis, Gallet et al. conclude that cholesterol is required for long-range signalling, perhaps by targeting Hh to the apical surface from where its long-range transport would be launched. This shortened range of Hh in the absence of cholesterol is at odds with the long-range Hh transport that is predicted from the finding that HhN^{palm} expressed in the P wing disc compartment activates *dpp-lacZ* expression throughout the A compartment (Burke et al., 1999). In support of this latter result, Callejo et al. report the widespread activation of two low-level Hh targets, *dpp-lacZ* and *iro*, around clones of cells overexpressing GFP-tagged HhN^{palm} (Callejo et al., 2006). Dawber et al. (Dawber et al., 2005) also confirm this finding with an untagged HhN^{palm} variant. In their study, they use two other low-level target genes, *ci* and *col*, as a read-out for Hh signalling. The three reports agree that a lack of cholesterol leads to a reduction in the activation of high-level Hh targets, such as *en* and *ptc*, and a concomitantly shortened range over which these target genes are activated. However, there is a clear disagreement as to whether HhN^{palm} activates low-level Hh targets over an extended or reduced range. How could this be explained?

The range of HhN^{palm} could depend on where it is made

Gallet et al. find that isolated HhN^{palm}-producing clones in the peripodial epithelium seem to activate the pathway over a long range, whereas isolated clones expressing HhN^{palm} in the columnar epithelium act at a short range (Gallet et al., 2006). In their view,

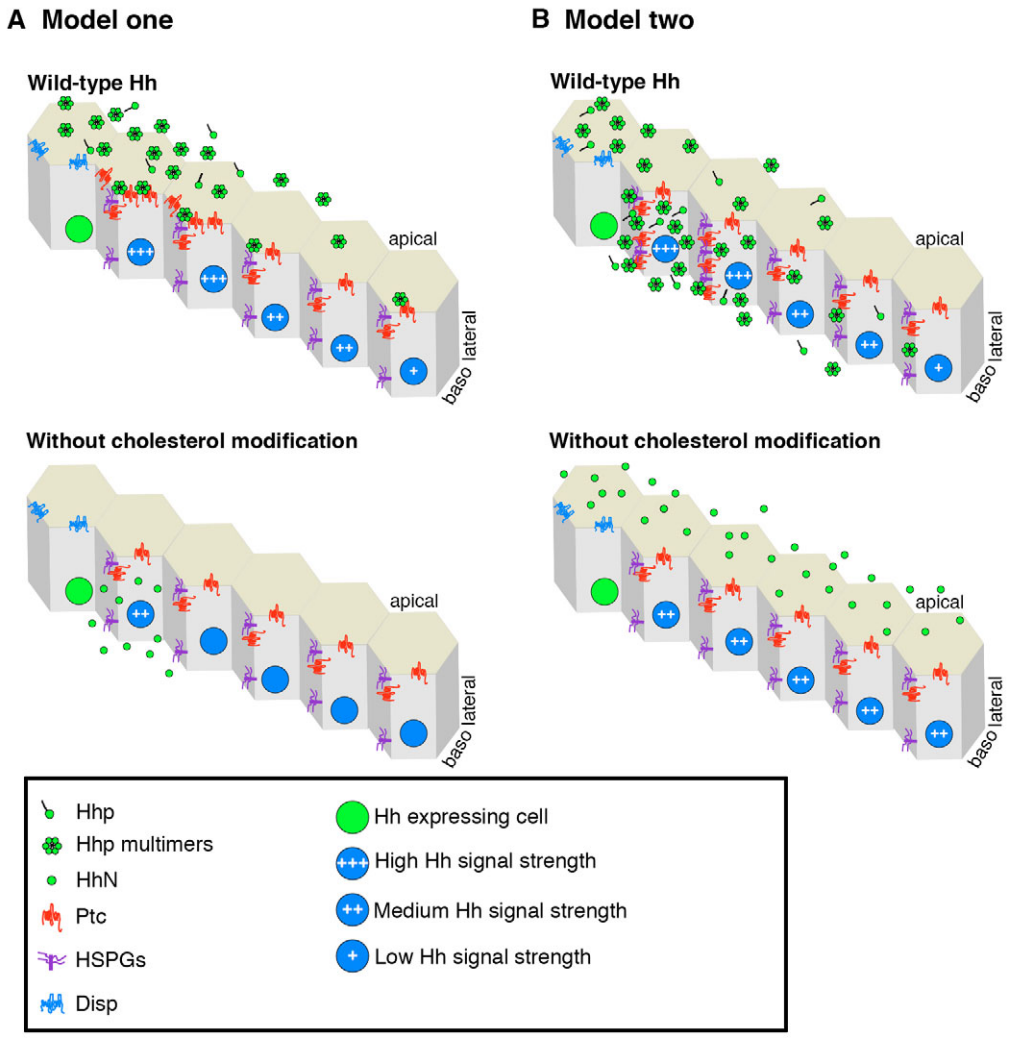

Fig. 3. Two models for Hh transport. A schematic of wing disc epithelia. (**A**) In model one, Gallet et al. suggest that wild-type Hh is normally secreted and released by Disp on the apical side (Gallet et al., 2006). In the absence of cholesterol, Hh could only be secreted basolaterally, where it would remain confined near the source. If, however, HhN^{palm} is expressed from peripodial cells (not shown in the diagram), it would flood the peripodial space (at the apical side of the columnar epithelium) and activate low target genes throughout. (**B**) By contrast, in model two and according to Callejo et al., Hh is also normally secreted and released by Disp on the apical side. However, according to their view, in receiving cells, Hh is normally present in the basolateral space, where it is trapped by either Ptc or HSPGs (Callejo et al., 2006). The binding of Hh to Ptc leads to high threshold target expression and to the sequestration of Hh near its source. Binding of Hh to HSPGs could lead to further transport. Without cholesterol modification, Hh is secreted in a Disp-independent manner on the apical side. There it would be free to spread throughout the disc, but would only activate low-level target genes, possibly via a distinct set of receptors. Because Ptc and HSPGs are not involved in the retention of HhN^{palm}, no gradient can be formed.

earlier studies failed to consider that Hh produced from peripodial cells could behave differently from Hh made by columnar cells (although Callejo et al. were aware of this concern). Indeed, Flp-Out clones are, by nature, induced randomly, and do appear in the peripodial epithelium at some frequency. These can easily be overlooked because they are in a different focal plane from columnar clones. The presence of one such clone could give the erroneous impression that columnar clones in the same disc have a long-range effect. The model of Gallet et al. (see Fig. 3A) suggests that, in the columnar epithelium, Hh trafficks to the apical surface, where it would normally be processed by Disp for release, and then subsequently transferred to the transport machinery (Gallet et al., 2006). Because of the absence of cholesterol, HhN^{palm} would hardly reach the apical surface. Instead, it would be released in a Disp-independent manner from the basolateral surface (which lies on the exterior of the disc), where it would fail to access the apically located long-range transport machinery and would eventually become diluted in the haemolymph. According to Gallet et al., peripodial cells would secrete HhN^{palm} equally towards both surfaces. A significant amount would therefore reach the peripodial space, where it would be free to diffuse and activate *dpp* expression in competent cells (throughout the A compartment of the columnar epithelium; Fig. 3A). Callejo et al. agree that peripodially produced HhN^{palm} acts at a long range (Callejo et al., 2006). However, they find that this is also true for HhN^{palm} produced from the columnar cells. This is based on the use of peripodial and columnar-specific Gal4 drivers to express the various forms of Hh (although the tissue specificity of the drivers was not rigorously verified). Overall, the model of Callejo et al. (see Fig. 3B) is that $HhN^{palm;choles}$, which has high signalling activity, is strongly retained at the basolateral surface of columnar cells near the source of expression. It would therefore activate high-level target genes over a short range. Because of the absence of cholesterol, HhN^{palm} does not accumulate at the basolateral surface (owing to the lack of an appropriate trafficking signal) and, because it has reduced affinity for the cell membrane, it would be released on the apical side into the peripodial space. Such a form of Hh would only activate low-level target genes, albeit at a long range. In addition, Callejo et al. show that cholesterol-deficient HhN^{palm} is internalised by cells throughout the wing disc (consistent with long-range spread), both in a Ptc-independent and Ptc-dependent manner (Callejo et al., 2006). They suggest that two modes of Hh sequestration and degradation could be at work. The Ptc-dependent mode would ensure a self-limiting restriction of the range of highly active multimeric Hh, as *ptc* expression is upregulated by high-level signalling. In parallel, Ptc-independent internalisation of cholesterol-deficient HhN^{palm} would function throughout the wing disc irrespective of Hh signalling. Because such a system would not be activated by Hh signalling, it would not be upregulated near the source and would thus allow a wider distribution of Hh.

In short, although there is agreement that wild-type Hh originating from the peripodial epithelium acts over a long range, there is a clear disagreement on the actual range of cholesterol-deficient HhN^{palm} when it is expressed in the columnar epithelium. Gallet et al. report that, when produced by columnar cells, HhN^{palm} acts at a reduced range (Gallet et al., 2006). By contrast, Callejo et al. and Dawber et al. report that HhN^{palm} produced by columnar cells acts over an extended range (Callejo et al., 2006; Dawber et al., 2005). There is no obvious explanation for the difference. However, we note that the clones shown by Gallet et al. tend to be small relative to the ones shown by Callejo et al. and Dawber et al. Small clones have a large perimeter-to-surface ratio and are therefore surrounded by a large number of wild-type cells, relative to the amount of Hh produced. For such clones, Ptc-dependent internalisation is expected to trap all the Hh produced and thus prevent further transport. When the relative amount of Hh produced increases (because clones are bigger or Hh is expressed at a higher level), the Ptc-dependent mechanism might become saturated, allowing unsequestered Hh to travel further. Ptc-dependent endocytosis might be saturated more readily by HhN^{palm} than it is by wild-type Hh, as HhN^{palm} does not feed into the Ptc-mediated negative-feedback loop and therefore does not enhance Ptc expression. This would explain why large clones that express wild-type Hh would allow Hh to act at a shorter range than does HhN^{palm} produced by similarly sized clones. Differences between the experimental results could also arise from differences between the detection protocols (such as the time allowed between clone induction and staining), or differences between the constructs. The exchange of the reagents and protocols used in the studies will ensure that these data can be directly compared.

None of the three reports discussed above directly addresses whether unlipidated Hh exists in nature or whether lipidated Hh alone can account for the range of Hh signalling that is observed. One argument against the natural existence of HhN^{palm} is that low-level target genes are not activated in *disp* mutants (Burke et al., 1999; Dawber et al., 2005). Because Disp is not required for the release of HhN^{palm}, one would expect *dpp* expression to be unaffected by the absence of *disp*. It therefore seems that only $HhN^{palm;choles}$ is made and is capable of activating the full complement of target genes.

The role of heparan sulfate proteoglycans

In the absence of *tout-velu* (*ttv*), which encodes an enzyme that is required for the biosynthesis of HSPGs, Hh can no longer act at a distance from its source (Bellaiche et al., 1998). One likely interpretation of this finding is that HSPGs contribute to the tethering of Hh to the plasma membrane of receiving cells, thus preventing it from escaping the epithelium and allowing its further transport or Ptc-dependent sequestration. Indeed, in the absence of *ttv* activity, Hh can only be detected in cells adjoining its source. By contrast, internalised HhN^{palm} can be detected throughout *ttv* mutant clones, suggesting that cholesterol-deficient HhN^{palm} does not require HSPGs for its membrane retention (Callejo et al., 2006). This result opens up the possibility that cholesterol could be important for the interaction of Hh with HSPGs, maybe with the help of the secreted protein Shifted (Glise et al., 2005; Gorfinkiel et al., 2005). Such interactions could be further tested and investigated in cell culture. As in *Drosophila*, vertebrate glypicans have been suggested to act as co-receptors for Shh in cell culture assays (Carrasco et al., 2005). Another component of the extracellular matrix, a secreted HSPG (perlecan) has been proposed to act as a negative regulator of Shh signalling by controlling the amount of Shh available for binding to Ptc (Carrasco et al., 2005; Datta et al., 2006).

Conclusion

These recent papers highlight the importance of cholesterol in modulating the range and activity of Hh. However, readers are faced with conflicting results and models. In this review, we have attempted to highlight the differences in the hope that they can be resolved in the near future. First, the groups of Guerrero and Therond differ in their views on apicobasal localisation of Hh and its variants (Callejo et al., 2006; Gallet et al., 2006). This is relevant because the different views lead to distinct models of the route followed by Hh along the epithelium. There is also a clear

disagreement on the actual range of cholesterol-deficient HhN^{palm}. Gallet et al. report that HhN^{palm} acts at a reduced range (when expressed in the columnar epithelium) (Gallet et al., 2006). By contrast, Callejo et al. and Dawber et al. report an extended range for non-cholesterol modified Hh (Callejo et al., 2006; Dawber et al., 2005). These different experimental results are probably attributable to differences in the specific protocols, and further comparisons among the different groups will be needed to resolve the differences. Hopefully, the resolution of these differences will allow researchers to then integrate data from the study of Shh in vertebrate systems. So far, data from vertebrates seem consistent with the view proposed by Callejo et al. (Callejo et al., 2006): ShhN (no cholesterol) acts over a long-distance along the anteroposterior axis of the mouse limb bud, suggesting that the cholesterol moiety restricts the spread of Shh in this system (Li et al., 2006). Significant further progress will require more than resolving the differences outlined here. We believe that new approaches are needed to answer questions about Hh trafficking in vivo. For example, better pulse-chase experiments could be devised to track the various forms of Hh as they undergo secretion transport and endocytosis. Ideally, these experiments should be performed with a level of expression that is comparable to that of the endogenous gene. Overall, it is hoped that a better understanding of the mechanism that regulates the activity of Hh and Shh will lead to new ways of controlling it in a therapeutic setting.

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