Seminars in Cell & Developmental Biology 33 (2014) 52–62

Contents lists available at ScienceDirect

Seminars in Cell & Developmental Biology

journal homepage: www.elsevier.com/locate/semcdb

Review

# Hedgehog and its circuitous journey from producing to target cells

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ARTICLE INFO

*Article history:*
Available online 30 June 2014

*Keywords:*
Hh gradient
Hh processing
Hh recycling
Cytonemes

ABSTRACT

The hedgehog (Hh) signaling protein has essential roles in the growth, development and regulation of many vertebrate and invertebrate organs. The processes that make Hh and prepare it for release from producing cells and that move it to target cells are both diverse and complex. This article reviews the essential features of these processes and highlights recent work that provides a novel framework to understand how these processes contribute to an integrated pathway.



**Contents**



## 1. Introduction

Hedgehog (Hh) was identified by genetic screens in *Drosophila melanogaster*, and much of our understanding of its roles and of the mechanisms involved in Hh signaling has come from studies in the fly. *hh* received its name from the phenotype of mutant *Drosophila* embryos, which have a lawn of disorganized hair-like cuticular

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http://dx.doi.org/10.1016/j.semcdb.2014.06.016
1084-9521/

protrusions reminiscent of a hedgehog's spines [1]. Subsequent findings show that Hh has many roles and is not dedicated only to embryo segmentation. Indeed, Hh is essential for most organs in the fly and for many metazoan tissues, and in many of these contexts it appears to function as a morphogen, spreading from the cells that express it to trigger differential, concentration-dependent responses in target cells. The mechanism by which it spreads is both fascinating and important to understanding its function.

In mammals, the Hh protein family has three members: Sonic Hh (Shh) [2, reviewed in 3], Indian Hh (Ihh), and Desert Hh (Dhh). Shh has multiple roles and appears to function in several modes. In the ventral neural tube, it induces distinct neural fates in a concentration-dependent manner [reviewed in 3]. A temporal gradient of Shh gradient has been implicated in the specification of digit identities [4,5]. And in the developing nervous system, there is evidence for a role of graded Hh activity in the control of axon guidance such that the growth cone and axon respond to the gradient slope rather than to absolute concentration [6]. These findings suggested a model in which the Shh gradient initially patterns the ventral spinal cord, acting as a morphogen, and subsequently functions in axon guidance [7–11]. Although the signaling pathway that directs growth cone turning may differ from the Hh pathway in other contexts [12], Hh appears to function as a paracrine, non-autonomous signal in all contexts. Ihh and Dhh also appear to be paracrine signals. Ihh negatively regulates the differentiation of proliferating chondrocytes in the appendicular skeleton [13,14]. Dhh regulates the male germline [15–17].

A key feature of the Hh protein is its lipophilicity. It is modified with cholesterol and palmitate, and as a consequence has high affinity for membranes. Despite this, in many contexts Hh travels many cell diameters from the cells that produce it, for example up to 50 μm (30–40 cells) in the *Drosophila* wing imaginal disk. In this organ, Hh is expressed by posterior (P) compartment cells and distributes to form a concentration gradient that spans approximately 8–10 anterior (A) compartment cells from the A/P compartment border (Fig. 1). Recent findings now show how Hh moves to generate such concentration gradients [18,19]. Much prior work has identified and characterized the processes that produce, disperse and receive Hh, showing that these processes are complex and involve the contributions of many different proteins. Although fundamental aspects of these processes remain to be elucidated, our purpose in this review is to present what is known about them and to describe how these processes relate to and can be understood in the context of the mechanism that generates Hh gradients.

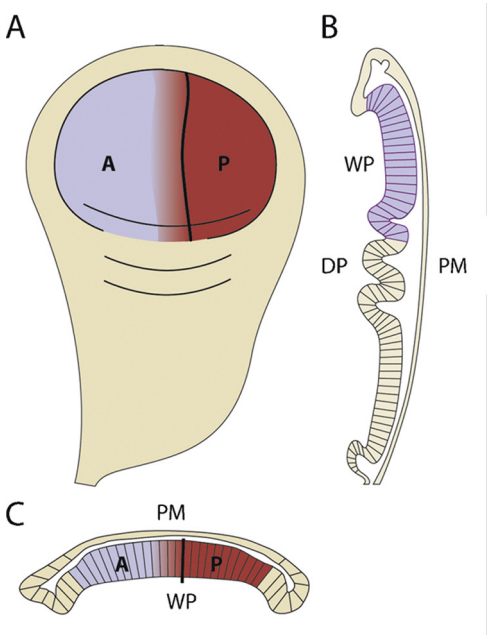


**Fig. 1.** Schematic representation of the epithelial layers of the *Drosophila* wing imaginal disk. (A) Frontal representation of the disk proper (DP) of the wing imaginal disk. The A compartment of the presumptive wing region (wing pouch) is indicated in blue and Hh expression in the P compartment in red. (B and C) Longitudinal section (B) and cross-section (C) of the wing disk showing the Peripodial Epithelium (PE) and DP epithelium. The wing pouch is also labeled as (WP) in (B and C). The Hh gradient at the A/P compartment border is graded red, and a thick line marks the A/P compartment border in (A, C). The schematic representation does not depict the exact cell numbers in the wing disc.

## 2. Hh production

### 2.1. Hh processing

Post-translational processing of Hh removes N-terminal signal sequence residues (1–84 of Hh, and 1–23 of Shh) and attaches palmitate by a stable amide linkage to the N-terminal cysteine of the N-terminally truncated *Drosophila* Hh (c85) and vertebrate Shh (c24) proteins [20–22]. Palmitate addition is catalyzed in the endopasmic reticulum (ER) by membrane-bound O-acyltransferases [23]. In *Drosophila*, the acyltransferase encoded by *rasp* (also known as *sightless*, *skinny hedgehog*, and *central missing*) is required in cells that produce Hh (Fig. 2, right panel) [21,24–26]. Skn, the murine homolog of *rasp*, is essential for Shh activity and for generation of the Shh protein gradient [27], and the purified human Hhat, a Rasp homolog, palmitoylates Shh in cells and in vitro [20,28].

Hh also undergoes autoproteolytic cleavage in the ER, splitting into two parts to generate a modified N-terminal fragment (HhNp), which is linked to cholesterol, and an unmodified C-terminal fragment (HhC) (Fig. 2, right panel) [29]. Much evidence testifies to the importance of this post-translation processing and to the signaling functionality of the HhN domain. For example, mutations in human Shh that block cleavage and modification are associated with holoprosencephaly, a congenital malformation [27,30–32]. However, there is also evidence suggesting that Hh retains activity even if it has a defective protease active site (e.g., Hh-h329a) and has not undergone autocleavage and is not modified with cholesterol [33,34]. Recombinant uncleaved protein (HhU) activates signaling in cultured vertebrate cells, and HhU that is ectopically-expressed in fly eye imaginal discs partially rescues *hh* mutant phenotypes in the eye [34]. Nevertheless, it has not been established that HhU is present or is an active paracrine signal under normal conditions. Shh autoprocessing is inhibited by depletion of sterols [35]. Unprocessed Hh molecules are targeted for degradation by the ERAD (ER-associated degradation) cascade; this mechanism could affect the levels of Hh in the mutants with impaired processing (Fig. 2, right panel) [23].

The protease active site that catalyzes autoprocessing is located in the HhC domain. It has a catalytic histidine (h329), and its activity and structure are homologous the processing domains of inteins [36, reviewed in 37]. After cleavage, the C-terminal domain is rapidly degraded in the ER lumen by the ERAD pathway [23]. HhC may have additional functions. Studies of Hh signaling in the *Drosophila* eye suggest that the C-terminal domain targets Hh to axons and growth cones of photoreceptor neurons [38].

### 2.2. Roles of the Hh lipids

The roles of the palmitate and cholesterol modifications have been investigated by expressing altered forms of Hh proteins that either lack palmitate but have cholesterol (Hh-c85s, Shh-c25s), that have N-terminal palmitate but lack cholesterol (HhN, Hh-h329a, ShhN), or that lack both palmitate and

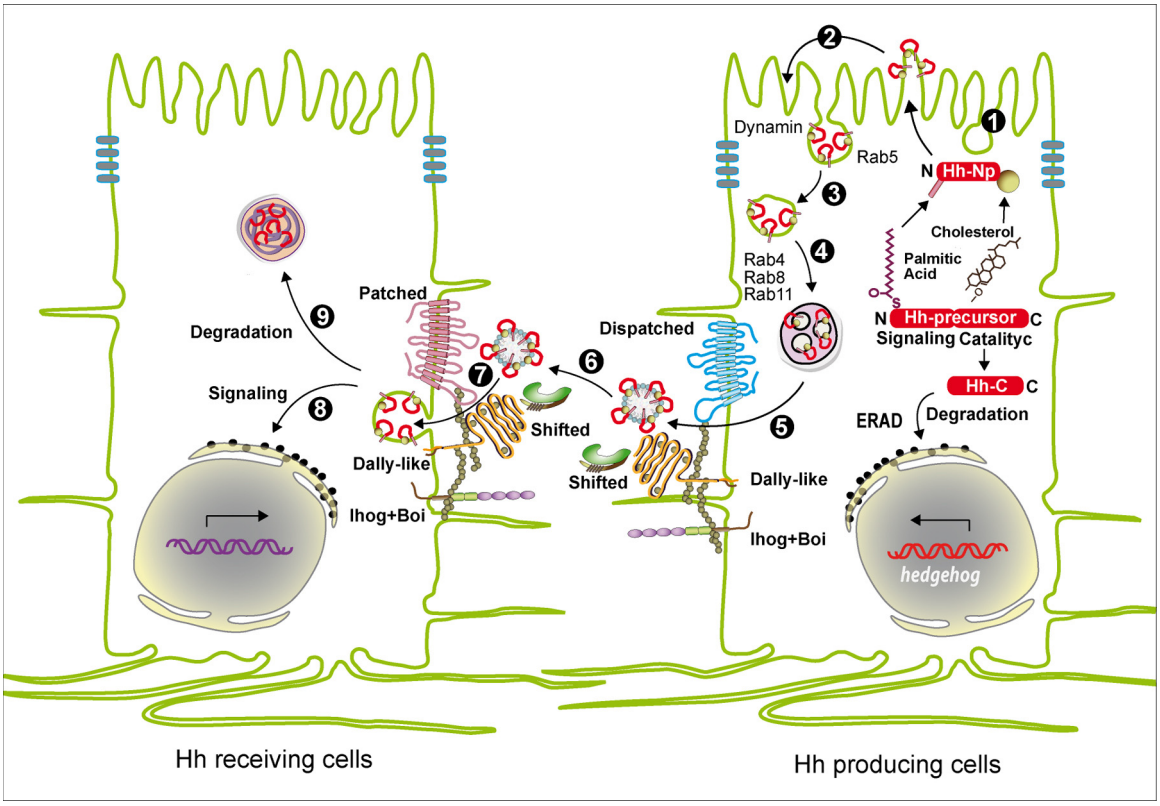


**Fig. 2.** Hh processing, trafficking, release, and reception in the *Drosophila* wing imaginal disk. In these schematic representations of a Hh producing cell (right panel) and a Hh receiving cell (left panel), steps in Hh production, processing, movement and reception are depicted and enumerated. (1) Hh transcription, processing, lipid modifications and apical externalization; (2) internalization mediated by (3) dynamin and Rab 5; (4) recycling mediated by Rab 4, 8, 11 to form multivesicular bodies; (5) release mediated by Disp, Dlp, Ihog, Boi; (6) transport mediated by Ihog, Boi and Shf; (7) the receptor complex of Ptc, Dlp, Ihog, Boi; (8) lysosomal degradation; and (9) signal transduction and target activation.

cholesterol (HhN-c85s, ShhN-c25s). In addition, fly and mouse mutants have been studied that lack the acyltransferases that palmitoylate Hh and Shh. Studies of ectopically expressed mutant proteins and of mutant animals defective in palmitylation show that Hh protein that is not lipidated neither signals nor disperses normally [20,21,23,27,28,34,39–50].

In *Drosophila*, Hh that lacks palmitate, either because its acceptor site is mutated (Hh-c25s) or because the Rasp palmitoyltransferase is defective (e.g. in *rasp* mutants), has no measurable functionality in embryos or imaginal discs [21,25,39,40,44]. In some contexts, the presence of nonpalmitoylated Hh appears to reduce the function of co-expressed wildtype Hh [44]. Production of nonpalmitoylated Hh appears to be similar to wildtype protein because the amount of Hh and Hh-c85s present under conditions of ectopic expression is comparable [39]. However, the form and distribution of wildtype and mutant protein are not. Wildtype Hh exists both as a monomer and in higher molecular weight complexes due to either multimerization [51] or binding to other components [39,42]. Although both monomeric and multimeric Hh-c85s was detected in extracts of cultured S2 cells [27], only the monomer form of Hh-c85s has been detected in extracts of salivary glands [39].

Nonpalmitoylated Shh is functional in vivo, although its apparent signaling activity is less than palmitoylated forms. In cultured cell assays, it is approximately 800 fold less active than wildtype Shh [22,47]. In mouse systems, ectopic overexpression of nonpalmitoylated Shh induces gain of function phenotypes that are less severe than phenotypes produced by comparable amounts of wildtype protein [27,44,48], and of loss of palmitoyltransferase activity in *Skn* mutants causes defects that are characteristic of defective Shh signaling but are less severe than those in *Shh* mutants [27]. Whereas both monomeric and multimeric forms of Shh are present in cultured cells [27,51], nonpalmitoylated Shh does not form multimers. And in contrast to WT, Shh protein does not disperse measurably from it sites of synthesis in *Skn* mutants that lack palmitoyltransferase (*Skn*) or that express only Shh-c25s [27]. Proposed functions for the palmitate include increasing N-terminal hydrophobicity [52] and serving as a recognition substrate for a protease that releases Shh from the cell surface by "shedding" [49].

In normal wing discs, Hh disperses over 8–10 anterior (A) compartment cells and distributes in a concentration gradient that declines with increasing distance from the compartment border (Fig. 1). Over-expression conditions for Hh and Hh-c85s generate similar amounts of protein, suggesting that lipidation does not influence efficiency of synthesis [21,39,40]. However, distribution patterns of wildytpe Hh and Hhc85s differ. P compartment-specific expression of Hhc85s generates an anterior distribution that is sparse and is not graded [21,39].

Cholesterol linkage to the Hh C-terminus is an obligate step in the autoproteolysis reaction [29], and mutant forms that lack cholesterol have been generated either by inactivating the autoprotease activity (Hh-h329a) [33], which produces uncleaved protein (HhU), or by engineering a protein that terminates translation at the normal site of cleavage (HhN and ShhN). Noncholesteroylated Hh and Shh have signaling activity in cell culture assays and in vivo, but these forms of Hh and Shh have reduced signaling capacity and do not generate normal distributions in neighboring cells [33,34,39–43,45,46,53]. In *Drosophila* wing discs, over-expression conditions of Hh, HhN and Hhc85sN in the P compartment generate similar amounts of protein [21,39,40], but the noncholesteroylated forms distribute more broadly and uniformly than wildtype Hh [21,39,40,53,54]. These studies show that the lipid modifications are essential to gradient formation.

### 2.3. Hh secretion and dispersion require lipid modifications

The palmitate and cholesterol moieties increase Hh's affinity for membranes [42,55] and for raft lipid microdomains [27,56]. Release from cells is therefore unlikely to be spontaneous and constitutive, and lipophilic Hh is unlikely to move efficiently in an aqueous extracellular environment. The more likely possibility is that cells have dedicated processes that release Hh after it is synthesized and that facilitate its long-range movement.

Several types of mechanisms have been proposed to explain how Hh may overcome lipid-imposed confinement to the producing cell and how it may move efficiently to signal distant target cells. Genetic studies in both flies and vertebrates have identified *dispatched* (*disp*) as essential for Hh release and dispersion (Fig. 2, right panel) [24,57–60]. Disp is required only in Hh-producing cells, and in its absence, mutant cells retain cholesterol-modified Hh and signal only to adjacent cells. In contrast, *disp* mutant cells appear to secrete cholesterol-free Hh constitutively, and the released Hh signals at long range [57,60]. Vertebrate cells also require Scube2 [58,61–63] to release cholesterol-linked Shh into culture medium, but release of non-cholesterol-modified Shh is Scube2 independent [64,65]. Scube2 has a signal sequence and domains related to cubulin and to epidermal growth factor, and is thought to be a secreted glycoprotein. Although the nature of the activities of Disp and Scube2 and of their interactions with Hh are not known, Scube2 is proposed to capture Shh from a Disp-bound form [65]. Despite our incomplete understanding, the characterizations of Disp and Scube2 indicate that release of Hh is an active process and suggest that cholesteroylated and non-cholesteroylated Hh exit Hh-producing cells by different mechanisms. These observations have been interpreted to support the idea that cholesterol is a tether that must be severed to liberate Hh, but this model for a direct role of cholesterol may not be correct.

Another idea that has been investigated is that Hh moves between cells in a soluble form, despite its lipid modifications. Soluble Shh has been identified in conditioned media from both Shh-transfected cells and explants of chick limb buds [51]. Isolation of the soluble Shh revealed that it was not monomeric, as it migrated at six times its native molecular weight in gel filtration chromatography. Although the exact composition of these aggregates is not known, these observations have been interpreted as evidence that Hh is released in a form that masks its lipid moieties from aqueous contacts. Soluble Shh is also generated by limited proteolysis in cultured cells that over-express ADAM proteases [49,66]. Shh in the culture medium is deleted of its N-terminal residues, and the truncated protein may have greater solubility because it lacks palmitate. Association with the Lipophorin and extraction from membranes into lipoprotein particles is another proposed mechanism for release and movement of lipid modified Hh [67,68].

There is also evidence that the composition and features of the extracellular environment are critical to Hh dispersion and signaling, and it may be important in this context that they influence cholesterol-modified and non-cholesterol-modified Hh differently. In *Drosophila* wing discs, Hh that is both palmitoylated and cholesteroylated does not move or signal across cells that lack Heparan sulfate proteoglycans (HSPGs). In contrast, signaling by HhN, which has a more extended range than fully modified Hh, is not affected in mutants that have defective HSPGs [69–72]. HhN, which is palmitoylated but not cholesteroylated, apparently does not require the extracellular matrix (ECM) to disperse [39,69]. The requirement for the ECM by normal Hh has been interpreted as evidence that cholesteroylated Hh interacts with the ECM and that the role of cholesterol is to regulate extracellular movement in the context of the ECM. However, it is important to note that these experiments show only that the extended range and ECM-independence of HhN are consequences of the absence of cholesterol. Specifically, they do not rule out the possibility that there are essential processes in Hh-producing cells that involve the cholesterol moiety and that are unable to accommodate non-cholesteroylated HhN. The observed abnormal behavior of HhN may therefore be a downstream and indirect consequence of mishandling in the Hh-producing cell. As noted above, HhN does not exit producing cells by the normal mechanism.

A key insight has been provided by experiments that examined signaling between the columnar and peripodial epithelial layers of the wing disk, both of which express Hh. In normal discs, there is no apparent Hh signaling between the two layers, despite their close juxtaposition (2–6 μm), and Hh that is overexpressed in the peripodial epithelium does not induce responses in the columnar epithelium. However, peripodial cells that express non-cholesteroylated HhN do signal to the columnar layer [39,42]. The common theme from these and the studies described above is that in the wildtype, there is an active process that releases Hh from producing cells – a process in which both cholesterol and functions such as Disp, Scube2 and HSPGs participate – and that non-cholesterol-modified Hh escapes the controls that this process normally imposes.

### 2.4. Lipid modifications barcode Hh for intracellular trafficking

The concentration gradient of Hh that extends anteriorly from the A/P compartment border of the wing disk has several components. One is intracellular and others are "extracellular", a designation that is operationally defined based on a staining protocol that denies access to intracellular antigen by applying anti-Hh antibody to unfixed discs [73]. Analysis of such antibody-stained discs by light microscopy detects concentration gradients of Hh that are at or near the apical and basolateral surfaces of anterior cells. The resolution of light microscopy is insufficient to discriminate whether antibody-bound Hh is attached to the cell surface or is free of, but closely situated to the cell surface; and although the difference in distance may be small, the distinction can be critical.

The path that Hh takes from P compartment cells to generate the anterior concentration gradient has been investigated extensively [39,42,60,74–76]. Ayers et al., 2010 concluded that apically- and basolaterally-localized Hh in the A compartment arrives from separate apical and basolateral sources in P compartment cells. In their model, Hh released apically from P compartment cells moves in the luminal space and is taken up apically by A compartment cells to form the long-range Hh gradient, and a separate process releases and takes up Hh basolaterally for activation of high threshold targets at short-range. In contrast, the findings of Callejo et al. [60] and Bilioni et al. [76] are consistent with the idea that Hh in the A compartment cells may be supplied by a single, indirect route from the basolateral compartment of P compartment cells. This model is based on the observation that although lipid-modified Hh localizes to both the apical and basolateral membranes of Hh-producing cells, it moves from the apical to the basolateral membrane by a vesicular-based intracellular trafficking pathway and is released predominantly from the basolateral pool. At the apical membrane, Hh is exposed to the lumen but is not released, and internalization by Dynamin-dependent endocytosis captures Hh in intracellular vesicles that move to the basolateral plasma membrane (Fig. 2, right panel). Hh in A compartment cells is presumed to generate its basal, internal and apical distributions from protein taken up from this basolateral source.

The indirect route suggested by this "recycling" model has several profound implications. Because Hh is actively redistributed intracellularly, its various intracellular and extracellular pools represent kinetic intermediates. Therefore, the relative abundance at different locations may be a function of residence time, and the

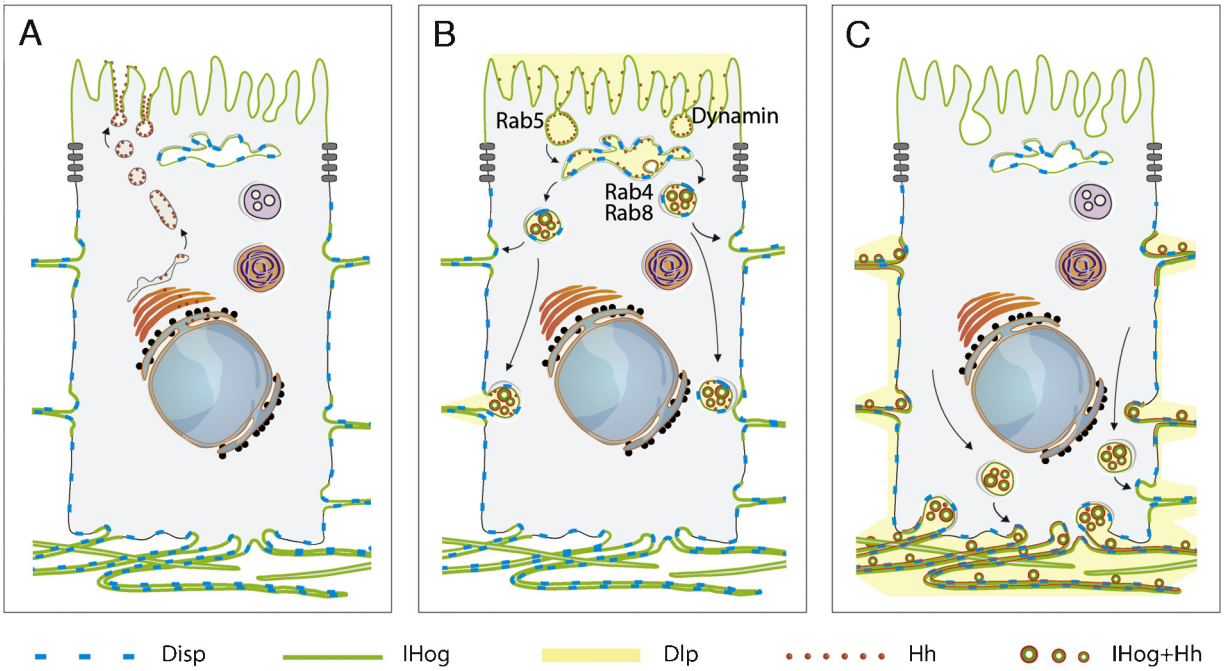


**Fig. 3.** The Hh recycling model. (A–C) Schematic representation of Hh trafficking in *Drosophila* wing disk epithelial cells prior to its release from producing cells. (A) Hh (red beads) is translated, cleaved, lipid modified and transported to the apical membrane. (B) Apical Hh is internalized in the same cells by a dynamin-dependent process, sorted to Rab5-containing endosomes and to Rab4/Rab8-containing recycling endosomes. Endosome-associated Hh interacts with Disp (blue patches on basolateral membrane), Dlp (yellow shade) and Ihog (green stretches on basolateral membrane). (C) Hh-containing multivesicular endosomes are transported to the basolateral membrane for association with cytonemes and for release in exosomes.

pathway that Hh takes in its journey from P to A compartment cells may not be revealed by its steady-state distributions. This issue pertains to the apical Hh in the A compartment, which may be directly supplied from apically-localized Hh in nearby P compartment cells [74], or may represent Hh that has taken a circuitous route involving redistribution for the apical membrane and basal release in P compartment cells, and basal uptake and intracellular translocation in A compartment cells [60]. Although real-time observation and pulse-chase analysis will be needed to fully delineate details of Hh movement, it is already clear that the distribution of Hh is regulated by intracellular trafficking (Fig. 2, right panel; and Fig. 3).

Non-cholersteroylated Hh and non-palmitoylated Hh does not redistribute normally from the apical to the basolateral compartment in P cells [39,60], appearing, instead, to concentrate apically in a diffuse, non-punctate form [29,39]. In contrast, the major fraction of wildtype Hh localizes in discrete puncta that are either apical, intracellular or basolateral. Hh's lipids may therefore be described as essential for targeting Hh to the pathway that captures Hh from the apical membrane and packages it in intracellular vesicles that move to the basolateral compartment. That is, cholesterol and palmitate may function together as a molecular barcode that provides entry to the intracellular trafficking pathway that prepares Hh for regulated release, and the apparently unrestricted spread of unlipidated-Hh through the apical lumen may therefore be a consequence of aberrant intracellular behavior that leads to abnormal apical release [60,76,77].

## 3. Preparing Hh for release

For this discussion, we enumerate three stages in the choreographed path that Hh takes prior to its release. The first involves the N- and C-terminal proteolytic cleavages, the palmitate and cholesterol modifications, and localization in the apical plasma membrane (Fig. 3A). It is not known whether and how these steps may be ordered, related or regulated, but protein that lacks either palmitate or cholesterol does not appear to participate in the subsequent processes that characterize the behavior of wildtype Hh [39,60]. Abnormal release into the apical lumen has been observed for Hh that lacks cholesterol or palimtate [39] and for wildtype Hh in cells that overexpress a mutant form of the glypican Dally [74,76]. Residence in the apical membrane may also involve association of lipid-modified Hh with lipid rafts [56].

In the second stage, Hh is captured from the apical membrane and concentrated in intracellular vesicles that move to the basolateral compartment [60]. Recycling of Hh is dependent on dynamin (*shibire* (*shi*)), which functions to generate endocytic vesicles from plasma membranes [reviewed in 78], and on Rab5, which functions in early endosomes [79]. Transcytosis of these Hh-containing vesicles is dependent on Rab4, Rab8, Rab11, Disp and the glypican Dally-like (Dlp) protein, and conditions that enfeeble or deplete these proteins change the distributions of Hh in ways that are consistent with an apical to basal pathway that prepares Hh for release (Fig. 2, right panel; and Fig. 3B). Mutant conditions for Rab11, Disp and Dlp also reduce the dispersion of Hh and reduce Hh signaling [18,24,41,57,59,60,80,81].

Rab4 and Rab11 regulate endosomal sorting and recycling in other contexts [82,83], and Rab8 participates in the recycling of cholesterol-containing endosomes [84,85], indicating that Hh transcytosis may be a type of endosomal cycling. Although Dlp has not been directly associated with endosomes in Hh-producing cells, Disp localizes to the basolateral plasma membrane and to subapical endocytic vesicles that appear to move basally. Some Disp-containing vesicles also contain Hh [60]. Dlp also appears to transcytose from the apical membrane to the basolateral compartment [86], and the co-immunoprecipitation of Dlp with Disp and recruitment of Dlp by Disp suggest that these proteins may colocalize in P compartment cells. Moreover, the similar but abnormal distributions of Hh in loss of function conditions for either Disp or Dlp supports the idea that they function in a common process [60].

The essential role of Disp for release of cholesteroylated Hh [57] and the requirement for Dlp support the idea that Rab4-, Rab11- and Rab8-dependent vesicles deliver Hh for basal release via a dedicated pathway of endosomal trafficking.

The third stage targets motile Hh-containing vesicles to basal cytonemes that transfer Hh between signaling cells [18,60]. These basal cytonemes emanate both from Hh-expressing and Hh-receiving cells, and both types of cytonemes appear to transport Hh in visible, punctate structures (Fig. 3C). Cytoneme-mediated signaling has been characterized most extensively in *Drosophila* wing discs and histoblasts; cytonemes emanating from Hh-expressing cells and populated with Hh-containing vesicles have also been described in the chick limb bud [87].

## 4. The cytonemes that mediate Hh transport

Cytonemes are specialized filopodia that mediate paracrine exchange between cells. They extend from cells that are active in cell-cell signaling in developing organs, from cells of the vertebrate immune system, and from virus-infected cells that transmit virus [reviewed in 88]. They have been most extensively characterized in *Drosophila* in contexts in which they have been implicated in the dispersion of the Hh, Decapentaplegic (Dpp), Fibrobast Growth Factor (FGF), and Epidermal Growth Factor (EGF) signaling proteins. These studies show that these signaling proteins are transported by different types of cytonemes, one of which transports Hh. The following brief description outlines their basic features.

Cytonemes have been observed that extend from both the apical and basal plasma membranes of polarized epithelial cells. They are actin-based, with an approximately 0.2 μm diameter. In the columnar wing disk epithelium, many are shorter than 5 μm, but apical cytonemes as long as 80 μm (>40 cell diameters) have been observed. Apical and basal cytonemes average approximately 20 μm and 12 μm, respectively, and predominantly orient perpendicular to the A/P compartment border [60,89]. In the abdomen, basal cytonemes extend from P compartment histoblasts into the A compartment and from A compartment histoblasts into the P compartment, orienting predominantly perpendicular to the compartment and segment borders (Fig. 4D and E) [18]. The average range is between 17–40 μm in length (4–10 cell diameters).

Several features of the apical cytonemes of the wing disk implicate these organelles in Dpp signaling. They are populated with motile vesicles that contain the Dpp receptor Thickveins (Tkv); they extend from cells in the wing blade primordium to make contact with Dpp-expressing cells at the A/P compartment border; and their distribution changes under conditions of reduced Dpp function or ectopic Dpp expression [89,90]. The wing disk basalolateral cytonemes, in contrast, mediate Hh movement. The basal cytonemes that extend across the A/P compartment border from the P compartment cells have motile vesicles that contain Hh and Ihog (Fig. 4B–E) [18,60], and those that extend across the A/P compartment border from the A compartment have motile vesicles that contain Hh, Ihog and Ptc [18]. Moreover, genetic conditions that deplete A cells of either Diaphanous (a formin) or SCAR (a regulator of actin polymerization) reduce the length and number of cytonemes and reduce both Hh signaling and the Hh gradient in the A compartment. The correlation between the basal cytonemes and Hh signaling also pertains to gain-of-function conditions: over-expression of Flotillin-2, a major component of membrane microdomains, increases cytoneme length and the extent of the Hh signaling domain.

As noted above, the wing disk cytonemes that have been implicated in Dpp signaling show apparent plasticity under conditions of altered Dpp expression or function [90], and cytonemes implicated in Hh signaling have also been found to be responsive to conditions

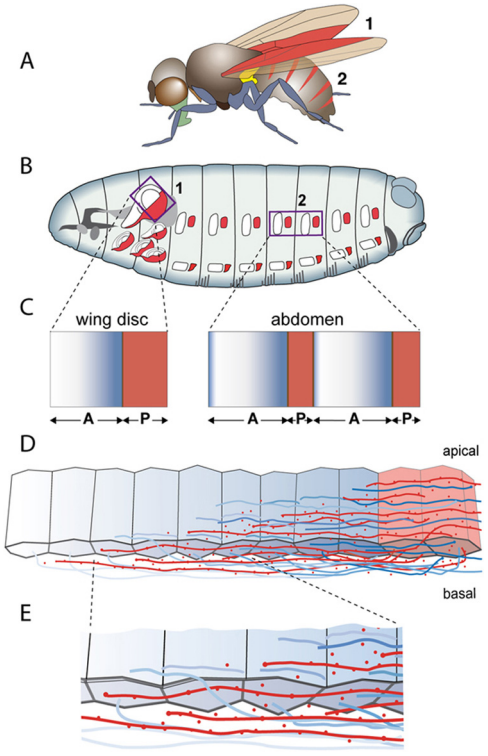


**Fig. 4.** Cytoneme-mediated Hh transport in *Drosophila* wing imaginal disk and histoblast epithelia. (A) Drawing of an adult *Drosophila* with various colors highlighting different body structures. (B) Drawing of a *Drosophila* larva showing several thoracic imaginal discs and abdominal histoblast nests, with their A and P compartments marked in white and red, respectively. Purple boxes outline the wing pouch of the wing disk (1) and the dorsal histoblast nests (2) of two segments. (C) The rectangles depict Hh-expressing (red) and Hh-signaling (blue) regions in the wing disk epithelium (left) and in two abdominal segments after the histoblast nest fusion (right). (D) Drawing of cells in the vicinity of the A/P compartment border of either disk or abdominal epithelia that express (red) or respond to (blue) Hh. Cytonemes from Hh producing cells (red) and from receiving cells (blue) vary in length and their dynamics are proposed to shape the Hh concentration gradient. (E) Enlargement of (D) to show exovesicles that contain Hh and move along cytonemes.

that change the landscape of Hh expression [89]. In the *Drosophila* ovary, where Hh signaling by somatic niche cells instructs escort cells that directly contact and maintain germ stem cells in an undifferentiated state [91,92], the lengths of Hh-containing cytonemes that extend from the somatic niche cells increases under conditions that compromise Hh signal transduction in escort cells [92]. And in the cells of the posterior histoblast nests, in vivo, real-time evidence for cytoneme-mediated Hh transport and cytoneme plasticity has been obtained [18]. These cytonemes have motile Hh- and Ihog-containing puncta. They are dynamic, extending to lengths that correlate with the domain of Hh signaling before retracting. These studies showed that cytoneme extension length and number correlates precisely in space and time with Hh gradient formation.

Hh signaling has been implicated in the formation of *Drosophila* embryonic gonads, which consist of primordial germ cells (PGCs) and somatic gonadal precursor cells. In this process, Hh is proposed to have a chemoattractant activity during PGC cell migration, and *disp*, *toutvelu* (*ttv*), and *shifted* (*shf*), which have been shown to be essential for Hh signaling and gradient formation in the wing disk [24,57–59,69,70,72,93,94], are also required for PGC migration [50,95,96]. Migrating PGCs have protrusions that orient in the direction of Hh signaling [96], but the published images and the lack of a proper analysis do not clearly reveal whether these structures are cytonemes.

In sum, histological and genetic data provide evidence for cytoneme-mediated Hh transport in several different contexts. The

data do not rule out other mechanisms of movement, but they show that signaling at the A/P compartment borders of several different tissues is cytoneme-dependent. Application of the GRASP (GFP reconstitution across synaptic partners) technique [97], which marks neuronal synapses [97,98], marks also the contacts that cytonemes make with target cells [99], and shows that the basal cytonemes at the wing disk A/P compartment border contact the cells they target. There are both structural and functional parallels between these contacts and neuronal synapses [reviewed in 100], supporting the idea that the transfer of Hh from producing to recipient cell is specifically at regions of organized contact that create a privileged environment that promotes efficient, controlled exchange. Given the role of Hh signaling in developmental patterning, growth and morphogenesis, and by analogy to neuronal synapses, we term these contacts morphogenetic synapses.

## 5. The Hedgehog transportation system

### 5.1. Proteins implicated in Hh transport

The circuitous path that is described by the recycling model of Hh signaling exposes Hh to multiple different contexts in producing cells (the apical membrane, endocytic vesicles, the basolateral membrane, and cytonemes), to the extracellular environment of the morphogenetic synapse, and presumably to a variety of domains in the recipient cells. It follows that there are many types of proteins that shepherd Hh along its journey, and also that the roles of these proteins may be difficult to establish because the effects on the Hh steady state distribution and on Hh signaling that are caused by changing their activities may be indirect. The following presents the known participants in Hh production, presentation and signaling from the perspective of this model (Fig. 2, right panel).

#### 5.1.1. Ihog and Brother of Ihog

Ihog and Brother of Ihog (Boi) are co-receptors that together with Ptc are required for high affinity binding of *Drosophila* Hh [101,102]. They are single-pass transmembrane proteins and are evolutionarily related to the vertebrate orthologs CAM-related/downregulated by oncogenes (CDO) and brother of CDO (BOC) [Okada, 141, p. 149 and reviewed in 103]. In *Drosophila* epithelial tissues, Ihog/Boi are expressed and required in both Hh-producing and Hh-receiving cells [76,102,104,105]. They localize to the plasma membranes, but with contrasting distributions: Ihog is mostly basolateral while Boi is mostly apical [60,76]. Under conditions of either Ihog or Boi over-expression, the amount of Hh in producing cells increases [60,76,101], while levels of Hh and of Hh signaling decrease in receiving cells [102,106]. Overexpressed Ihog marks and appears to stabilize the basal cytonemes, and its presence in basal cytonemes is consistent with a role in cytoneme-mediated trafficking [18,60,76]. Other Hh pathway components that have been detected in these cytonemes include Hh, Disp, Boi, Dlp, Dally and Shf [18,60,76]. Cytonemes in the chick limb bud that have Shh-containing vesicles also can be marked CDO-Cherry [87].

#### 5.1.2. Shifted

Shifted (Shf) is an ortholog of the vertebrate Wnt Inhibitory Factor-1 (WIF1) [76,93,94,107–109]. Although Wif1 functions in Wnt signaling in vertebrates [110,111], Shf has no apparent role in Wg signaling in *Drosophila*, but is necessary for dispersion and gradient formation of normal, cholesteroylated Hh. Its activity does not affect the release or distribution of unlipidated Hh [93,94]. In the *Drosophila* embryo, Shf expression in the gonadal mesoderm is required for Hh-dependent migration of the primordial germ cells [96]. In wing discs, it accumulates at the surfaces of Hh-expressing cells, and in wing discs lacking *shf* function, Hh basolateral localization and spreading are decreased [76,93]. Apparent interactions with Ihog and Boi suggest that Shf may interact directly with the Hh receptor complex [76,94,107]. In fact molecular and genetic interaction with Hh [93,94] and Dlp [108,109] has been reported. Shf spreads from cells that express it, appears to function non-autonomously [76,93,94,107,109], and its interactions with glypicans [76,108,109] suggest an ECM involvement. Thus, Shf appears to have an extracellular presence and to function in the process that disperses Hh, but we do not yet understand what its participation in Hh packaging and movement might be (Fig. 2, right panel).

#### 5.1.3. Scube2

Zebrafish Scube2 is proposed to be a secreted ECM protein that is necessary for normal levels of Hh transport and signaling activity [62,63,112]. It also appears to be specifically required for the release of lipid-modified Hh from Hh-producing cells [64,65]. This activity of Scube2 shows synergism with Disp, and the fact that Scube2 and Disp bind Hh independently suggests that release may involve a pass-on mechanism that transfers Hh from Disp to Scube2 [65]. Human Scube2 has also been shown to interact with Shh and to enhance signaling activity [113]. Although Scube2 appears not to be conserved in *Drosophila*, we can entertain the idea that a similar functionality may be provided by Shf, which also appears to be secreted and to contribute to Hh release [76,93,94,96,108].

### 5.2. The carriers that move Hh between producing and target cells

#### 5.2.1. Hh multimers

In many contexts, Hh moves over distances of many cell diameters despite the lipophilicity of its palmitate and cholesterol modifications, and several mechanisms have been proposed to enhance its solubility in an aqueous environment. As noted above, one proposed mechanism involves Hh oligomers, which have been detected in preparations of mammalian and *Drosophila* tissue culture cells, and may be a form that diffuses in the extracellular milieu of Hh-expressing tissues [27,42,51,114,115]. Oligomerization requires the palmitate and cholesterol modifications [27,39,42,115] and the conserved N-terminal domain [115], and in vitro, oligomerization requires transaglutaminase activity regulated by heparan sulfates [116]. Soluble Hh oligomers exhibit enhanced signaling activity in various assays, and can form concentration gradients [27,51,115,116]. Although these properties have favored the idea that Hh may be released and may disperse as a soluble oligomer, there is no in vivo evidence that it does. A contrasting role for oligomerization has been suggested by the findings that oligomerization is necessary for localization to lipid rafts [27] and that the lipid raft Flotillin-2 protein is required for Hh dispersion [117].

#### 5.2.2. Lipoprotein particles

A different class of model proposes that Hh associates with and is transported by lipoprotein particles [67,68,118]. The protein constituents of lipoprotein particles are likely to be lipophilic and to include proteins that are lipid-modified or GPI-anchored. In the wing disk, a small fraction of total Hh (~2%) co-purifies with and can be extracted with lipoprotein complexes [68]. Hh signaling is reduced under conditions that deplete lipophorin [68,118,119], a major protein constituent of lipoprotein particles [120,121]. Although short range Hh signaling appears to be undiminished and the effects appear to be limited to long range Hh signaling, it is not clear whether this difference reflects a quantitative response to lipophorin depletion, or if it distinguishes between mechanistically distinct signaling processes. In addition, because lipoprotein particles are involved in sterol delivery, it is possible that lipophorin depletion leads to a general sterol insufficiency that perturbs disk cells in ways that are not specific to Hh transport per se.

The results of many studies establish that Hh signaling at the wing disk A/P compartment border is activated by Hh that is produced by P compartment cells in the disk columnar epithelium (the "disk proper"). What then might be the significance of lipoprotein association? Lipophorin-containing lipoprotein particles circulate in the hemolymph after release from the *Drosophila* fat body where they are generated [120,121], and lipoprotein-associated Hh has been identified in larval hemolymph [67]. Hh that is associated with lipoprotein-particles has also been identified in a vertebrate system. Human Ihh (but not Shh or Dhh) co-purifies specifically with very low density lipoprotein (VLDL) particles, and this form was proposed to distribute Ihh systemically to the endothelium [122]. These findings raise the possibility that Hh may have systemic functions in addition to its role as a morphogen. In *Drosophila*, evidence that lipophorin crosses the blood–brain barrier and is necessary for neuroblast proliferation has been interpreted to indicate that lipoprotein particles may have roles that are independent of lipid delivery [123]. Thus, the question whether lipoprotein-association is relevant to contexts such as the Hh signaling gradient in the wing disk is important but not yet answered. The Hh signaling gradient requires cytoneme-mediated transport [18], and most likely release of Hh from producing cells and uptake by receiving cells at the synapses that form at sites of cytoneme contact. The state of the Hh protein that moves across the synaptic gap is not known, but is conceivably a vesicular form [124] that may include lipophorin.

#### 5.2.3. Extracellular matrix

Genetic studies have shown that Hh does not cross over patches of cells that are mutant for toutvelu (ttv), brother of ttv (botv) and sister of tourvelu (sotv) [69,71,72]. Because these genes encode proteins of the EXT family that are closely related to glycosyltransferases that synthesize heparan sulfate proteoglycans (HSPGs), models suggesting that Hh binds transiently to HSPGs as it moves over the surface of cells have been proposed [reviewed in 12]. The discovery that cytonemes mediate Hh transport suggests an alternative; that the absence of HSPGs inhibits cytonemes, which appear to track over the surface of cells. Indeed, experiments that monitored cytonemes in the vicinity of $ttv^{-/-}$ or $ttv^{-/-}$; $botv^{-/-}$ mutant clones showed that cytonemes do not cross over HSPG-defective cells [18]. These observations suggest that interactions with HSPG-modified cell membranes are essential for the extension or stabilization of the basolateral cytonemes that mediate Hh transport, and that the observed effects of HSPG-defective mutant clones on Hh transport and signaling may be indirect.

#### 5.2.4. Exosomes

Exosomes are 30–100 μm particles that circulate in the vertebrate vasculature and are proposed to have various roles, including signaling [reviewed in 125]. Although there is no direct evidence that they are involved in Hh signaling in *Drosophila*, signaling by Hh-related proteins in *Caenorhabditis elegans* is reduced in conditions that impair exosome secretion [126], and a vesicular form of Shh has been implicated in the establishment of left-right asymmetry in vertebrate embryos [127]. In *Drosophila* wing discs, vesicles marked with a fluorescence-tagged form of Ihog are present in Hh-producing cells as well as basolateral in regions where cytonemes are found (Fig. 4E) [18]. The absence of Ihog-marked vesicles in $ttv^{-/-}$; $botv^{-/-}$ mutant clones and in areas anterior to $ttv^{-/-}$; $botv^{-/-}$ mutant clones is consistent with the idea that these extracellular vesicles are specialized exosomes that transport Hh between Hh-producing and receiving cells [18]. Moreover, recycling of Hh from the apical membrane and packaging Hh in multivesicular endosomes may serve to prepare it for release in an exosomal form (Fig. 3A-C). The fact that the Hh activity gradient declines under conditions of RNAi-mediated down-regulation of genes involved in exovesicle production and/or release may therefore be understood in the context of Hh-containing vesicles that are released at the cytoneme synapse [18].

## 6. Hh reception and uptake

Hh-receiving cells express several types of proteins that are involved in Hh reception. One is Ptc, which has twelve transmembrane domains [128,129], is evolutionarily related to the RND family of channels and transporters [130], and binds Hh proteins [131–134]. The second type are the co-receptors Ihog and Boi, which are single pass transmembrane proteins of the immunoglobulin family that also bind Hh [102,106]. Both these types are required for Hh reception and signaling [102,106,135]. Mammals express an ortholog of Ptc [136], Cdo and Boc, which are essential for Hh signaling [137,138] and are orthologous to Ihog and Boi [101,139–143], and Gas1, a Hh co-receptor that is vertebrate-specific [137,138,144]. The third type is Smoothened (Smo), which is a seven transmembrane protein that is also essential for Hh signaling [145,146]. In the absence of evidence that Smo binds Hh directly, Smo is presumed to function downstream of Ptc, Ihog and Boi, and to indirectly relay Hh reception at the cell surface to the signal transduction apparatus in the cell (Fig. 2, left panel) [reviewed in 147].

In addition to Ptc, the immunoglobulin family co-receptors and Smo, Hh-receiving cells express several other proteins that are involved in Hh reception. Vertebrates express the Hedgehog-interacting protein Hip1, a membrane-bound glycoprotein that binds Hh [148]. Genetic loss-of-function and gain-of-function conditions for Hip1 suggest that it modulates Hh signaling by negative feedback [148]. Invertebrates do not express an orthologous function, but there is evidence in *Drosophila* of important modulatory roles for several proteins that target Hh extracellularly. Expression of the glypican Dlp is required for normal Hh signaling, appearing to increase Hh abundance and enhance internalization with Ptc (Fig. 2, left panel) [reviewed in 12]. Dlp activity is cell-autonomous, but does not require either heparan sulfate glycosaminoglycan (GAG) chain modifications [149], or binding to Hh or to an Hh:Ihog complex [150]; how or precisely where Dlp contributes to the process of Hh uptake and signaling has not been determined. Genetic perturbations implicate the glypican Dally in Hh signaling, but unlike Dlp, Dally is not essential [76]. It is only required for the extracellular levels and distribution of Hh.

Although GAG modifications do not appear to be required for Dlp to function, there are several observations that indicate an important role for the GAG chains in Hh signaling. DSulfatase-1 is an enzyme that hydrolyzes sulfates from GAG chains, and its function is needed for normal release of Hh from source cells and for Hh signaling in responding cells [151]. This suggests that sulfation may contribute to interactions between GAG chains and Hh (or other constituents of Hh signaling), and that Hh signaling is sensitive to the strength of these interactions. Another possible role for GAG chains has been suggested by the observation that Ihog dimerization and high affinity interactions of Ihog with Hh are induced by heparan [152]. The context that presents heparan to Ihog or other constituents of Hh signal transduction has not been identified. Results from studies of vertebrate systems similarly indicate an important role for glypicans and HSPGs in Hh signaling [153–157], but they also have not identified either the site or mode of action.

## 7. Concluding remarks

The impressive complexity and large number of steps that are involved in Hh production, dispersion and reception may be bewildering. Certainly they are puzzling if the expectation is that Hh's

movement is governed only by the laws of chemistry and mass action, and therefore that the observed distributions of Hh form spontaneously. By this view, Hh may be constitutively released by Hh-producing cells to form the requisite extracellular distributions that receptors present on the surface of responding cells process for signal transduction. If, however, the governing principle is understood to be that of precisely controlled targeting so that Hh dispersion is both directed and regulated, then the mechanism that moves Hh across target fields and generates the Hh concentration gradients must also provide both control and constraint. Cytoneme-mediated exchange at points of direct contact is a mechanism by which Hh released from producing cells is taken up by receiving cells at specific sites where the receptor is localized, and whose lifetime, selection and composition may be regulated. Recent work indicates that Hh dispersion in *Drosophila* wing discs and histoblast nests is cytoneme-mediated and that cytonemes and cytoneme synapses are essential for Hh gradient formation [18]. In this context, the many and varied constituents and processes that sculpt Hh's journey can be understood in the context of efficient but constrained delivery. The intent of this review is to show that the known features of Hh signaling are compatible with the cytoneme mechanism and to provide the reader with a framework to understand the many observations that have been made.

## Acknowledgements

Drs. Guerrero and Kornberg thank members of their respective laboratories, past and present, for their contributions to understanding the Hh gradient. We also thank J. A. Belio of the CBMSO by the artistic drawings for Figures. Dr. Guerrero was supported by the Spanish MINECO grant BFU2011-25987, the Marie Curie FP7 grant ITN238186 and by a CBMSO Institutional grant from the Fundación Areces. Dr. Kornberg was supported by NIH Grants R01-GM030637 and R01-GM110259.

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