# On the range of Hedgehog signaling

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Hedgehog (Hh) is a secreted signaling protein that regulates the development of many organ systems. It can travel from its site of synthesis, a process that involves covalent attachment of cholesterol to its carboxyl terminus, proteins with putative sterol sensing domains in both sending and receiving cells, and glycosaminoglycans. Understanding how the movement of Hh is controlled and propelled will be key to understanding how it carries out its essential roles.

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**Abbreviations**

| | |
|---|---|
| **BMP** | bone morphogenetic protein |
| ***disp*** | *dispatched* |
| ***dpp*** | *decapentaplegic* |
| **FGF** | fibroblast growth factor |
| **Hh** | Hedgehog |
| **Ptc** | Patched |
| **Shh** | Sonic hedgehog |
| **Smo** | Smoothened |
| **SSD** | Sterol-sensing domain |

## Introduction

If there is a code that universally specifies and regulates the growth and differentiation of organ systems in eukaryotes, it is based at least in part on the Hedgehog (Hh) protein. The *hh* gene was first identified as one of the many that are required for segmentation of the *Drosophila* embryo [1]. We now know that it is also involved in numerous other aspects of embryonic, larval and adult development in the fly and that it has homologs (e.g. Sonic hedgehog (Shh), Desert, and Indian Hh), that play key roles in human, mouse, frog, fish and chick development. The Hh proteins are secreted and are thought to function as morphogens, signals that elicit concentration-dependent responses from target cells. In this review, we describe what is known about the properties of Hh and the proteins that help it to function, then cite the evidence that Hh is used widely in both vertebrate and invertebrate development, and finally discuss the evidence that Hh can signal at long distances as well as short. It seems likely that the mechanisms of Hh signaling represent novel ways to regulate protein traffic in and outside cells.

## The Hh protein and its supporting cast

The Hh proteins acquire several post-translational modifications that strongly influence their behavior. *Drosophila* Hh protein is activated by carboxy-terminal cleavage and concurrent coupling to cholesterol [2,3]; the sequence and properties of the mammalian homologs are consistent with their undergoing similar reactions. The amino-terminal cysteine of mature human SHH is palmitoylated [4], and the conservation of the identity of this residue in all other Hh proteins suggests that palmitoylation may be common to these proteins as well. Both the amino- and carboxy-terminal modifications are likely to promote the observed tight association of Hh proteins to Hh-producing cells. They probably also mediate the association of Hh proteins with sphingolipid rafts [5••]. It is not obvious that these lipophilic properties of Hh are compatible with simple diffusion as a mechanism to move Hh from its site of production to its target.

Fascinating clues to the mechanisms that move Hh to its targets have come from the analysis of a *Drosophila* gene, *dispatched* (*disp*) that is required in Hh-expressing cells [6••]. Although Hh is synthesized and processed normally in *disp* mutant cells, Hh signaling is not. Interestingly, cholesterol-modified Hh (Hh-Np) fails to move from the *disp* mutant cells, whereas unmodified Hh (Hh-Nu) signals extensively without a need for *disp*. The Disp protein is predicted to have 12 transmembrane spanning domains and to be related phylogenetically to a family of proteins containing a sterol-sensing domain (SSD). Among the known SSD-containing proteins is Patched (Ptc), also a protein with 12 putative transmembrane spanning domains, and a Hh receptor. These properties underscore the importance that cholesterol is likely to have in directly influencing intercellular trafficking of Hh. The presence of sequences with homology to *disp* in expressed sequence tag databases of vertebrate cDNAs suggests that the cells that make the vertebrate Hh proteins also exploit the functions that Disp proteins provide.

Hh signaling is dependent upon the synthesis of proteoglycans in receiving cells and upon the gene *tout-velu* [7], which encodes a homolog of the vertebrate glycosyltransferases that are essential for the synthesis of heparan sulfate [8]. The role of the proteoglycans in Hh trafficking is not understood at present. Interestingly, heparan sulfate proteoglycan biosynthesis is also required for Wingless and FGF signaling, but these signaling pathways are not affected by *tout-velu* [9•].

Among the several Hh-binding proteins that have been identified, Ptc is thought to play a direct role in signal transduction. Vertebrate Ptc binds to Hh proteins [10,11] and is thought to mediate Shh signal transduction in cooperation with Smoothened (Smo). Smo is a transmembrane protein related to the secretin family of G protein-coupled receptors [12] but, although it is essential for signaling, it has not been shown to bind Hh directly. Rather, models have been proposed in which Ptc negatively regulates Smo, except when bound by Hh, and Smo signals constitutively except

**Table 1**

**Hedgehog-dependent organs and tissues.**

| Organ/tissue | Species | References* |
|---|---|---|
| *Drosophila* | | |
| Adult abdomen | – | [39,40] |
| Adult head | – | [41] |
| Bolwig's organ | – | [42] |
| Central nervous system | – | [43–45] |
| Embryo epidermis | – | [46–48] |
| Eye imaginal disc | – | [49–51] |
| Fat body | – | [52] |
| Female germline stem cells | – | [53] |
| Foregut and hindgut | – | [54] |
| Genital imaginal disc | – | [55] |
| Heart | – | [56,57] |
| Mesoderm/body wall muscles | – | [58] |
| Ommatidia/brain | – | [59] |
| Ovary border cells | – | [60] |
| Visceral mesoderm | – | [61] |
| Wing imaginal disc | – | [62,63] |
| Vertebrate | | |
| Anus | Mouse | [64] |
| Cartilage | Mouse, chick | [65,66] |
| Cerebellum | Mouse, chick | [67–69] |
| Eye | Mouse, zebrafish | [70–72] |
| Gut | Mouse, chick | [73,74] |
| Hair follicle | Mouse | [75,76] |
| Left–right asymmetry | Mouse, chick, zebrafish | [77–79] |
| Limb | Mouse, chick, zebrafish | [35,80,81] |
| Lung | Mouse | [82,83] |
| Mammary gland | Mouse | [84] |
| Muscle | Mouse, chick, zebrafish | [85–87] |
| Neural crest | Zebrafish | [88] |
| Notochord/floor plate | Mouse, chick, zebrafish | [30,89–91] |
| Pancreas | Mouse, chick | [92,93] |
| Peripheral nerve sheath | Mouse | [88] |
| Prostate | Mouse | [94] |
| Somite | Mouse, chick, zebrafish | [95–97] |
| Spinal cord or hindbrain motor neuron/interneuron | Mouse, chick, zebrafish | [98,99] |
| Testis | Mouse | [100] |
| Tooth | Mouse | [101,102] |
| Ventral forebrain | Mouse, chick, zebrafish | [89,103,104] |
| Ventrolateral midbrain | Mouse, chick | [105–107] |

*This reference list is not comprehensive but identifies relevant contributions made during the past several years only, and does not necessarily cite the initial observations.

when inhibited by Ptc (reviewed in [13]). It is not known how Ptc and Smo interact but recent evidence suggests that, in *Drosophila*, Ptc destabilizes Smo and that Hh induces internalization of Ptc and phosphorylation and stabilization of Smo [14•]. Analysis of Shh responses in mammalian cell lines suggests that Ptc may influence the conversion of Smo between an active and an inactive state [15•]. Neither of these observations requires that the action of Ptc on Smo be direct or that there be a stoichiometric relationship between Ptc and Smo.

Although there has been no demonstration that *Drosophila* proteins bind Hh directly, Hh protein can be found concentrated in discrete particles up to a distance of ~4–6 cells from *hh*-expressing cells in *Drosophila* embryos and wing imaginal discs [16]. It is not known if these particles correlate either with signaling or an endocytic disposal pathway or with a combination of both but Hh co-localizes with Ptc in these particles [6••,17]. The observation that Hh induces internalization of plasma-membrane-associated Ptc [14•] is also consistent with a direct interaction between Hh and Ptc. Unraveling the nature and significance of these observations awaits careful biochemical analysis.

## The many sites of Hh signaling

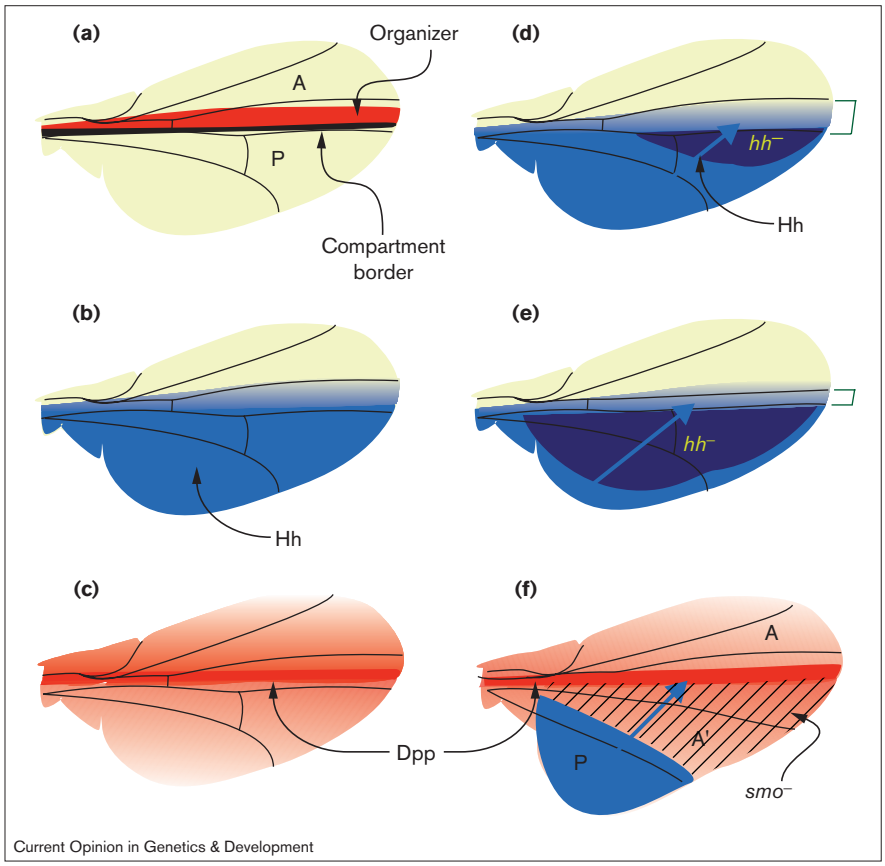
Since the *Drosophila hh* gene was cloned in the early 1990s, studies on *hh* have proliferated and are now represented by more than >750 references in the Medline database. We have learned most about the principles of Hh signaling from studies of the *Drosophila* wing imaginal disc, the chick limb and chick neural tube explants, and these studies will be described further in the next section. To illustrate the wide range of systems in which Hh signaling plays an important regulatory role, Table 1 presents the current list of tissues in which the role of Hh has been documented and analyzed. We assume that this list will continue to grow as the function of Hh is characterized further.

## Long-range Hh signaling in *Drosophila*

In *Drosophila*, Hh has been shown to signal at long-range in the developing adult eye and wing. Hh produced in retinular cells of the developing ommatidia is transported in retinal axons and delivered to post-synaptic target sites in the brain [18,19]. The distance from the retinular cell body to the brain varies from ~80–160 μm, but for Hh, only a small portion of that distance is apparently traveled outside of the cell. In the wing, Hh is produced by all of the posterior compartment cells and its immediate targets in the anterior compartment are the cells that lie close to the anterior/posterior compartment border (Figure 1b). Anterior cells up to 4–5 cells from the border upregulate the Hh target gene *ptc*, and those 4–10 cells from the border express *decapentaplegic* (*dpp*), the BMP homolog and Hh target gene that directs growth and patterning of the cells in both compartments. Dpp embodies the activity of the developmental organizer induced at the compartment border (Figure 1c), and we believe that the primary role of Hh signaling in this region is to induce it. Although the immediate proximity of posterior compartment Hh-producing cells to the anterior cells at the border suggests that Hh produced by the cells near the border may suffice, several observations indicate that cells far from the border can contribute Hh as well.

First, cells that lack Disp, the protein that is needed for Hh-producing cells in the wing disc to send Hh to target cells, accumulate Hh to abnormally high levels [6••]. This observation implies that the level of Hh normally present in posterior cells represents an equilibrium

**Figure 1**



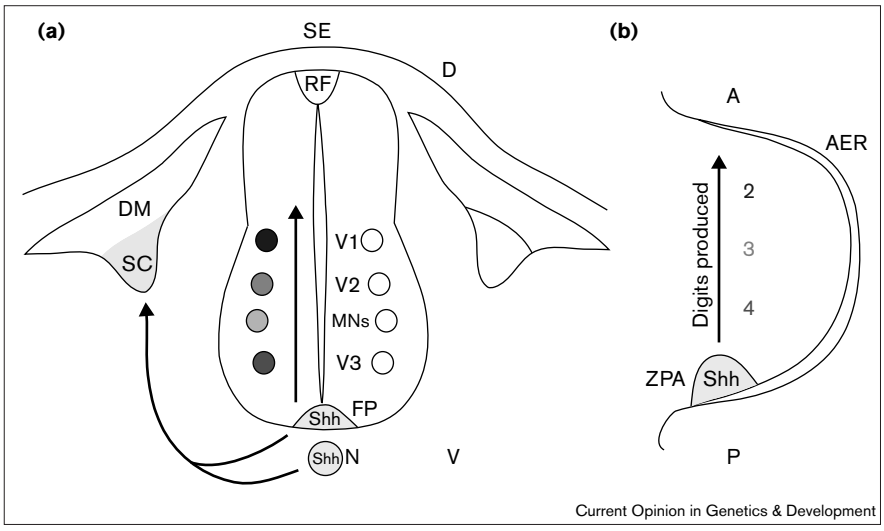

Signaling and organization that generates the *Drosophila* wing. **(a)** The wing is subdivided into anterior (A) and posterior (P) compartments by the anterior/posterior compartment border, which coincides approximately with the developmental organizer. **(b)** Hedgehog (blue) is produced in the posterior compartment and moves across the compartment border, a distance of 5–10 cells. **(c)** Dpp (red) is made by anterior compartment cells adjacent to the compartment border and is believed to move across the entire wing primordium. **(d,e)** Clones of *hh*⁻ cells (purple) do not interfere with the signaling that generates the organizer, although large clones do reduce growth in the middle of the wing (green brackets). **(f)** A large anterior *smo*⁻ clone (cross-hatched area) generates a mirror-image duplication of the anterior compartment (A′) that displaces the posterior compartment but does not block Hh signaling.

between synthesis and export, and that the level rises in *disp* mutant cells because of an export defect. As Hh levels are uniformly elevated in the posterior compartment of *disp* mutant wing discs, we might conclude that the rate of export is normally uniform and does not change substantially with distance from the border.

Second, clones of *hh*⁻ cells can grow to populate a significant proportion of the posterior compartment without affecting wing development, even if the clones abut the posterior side of the compartment border (Figure 1d; from T Tabata, TB Kornberg, unpublished data). *dpp*-expressing cells depend upon Hh signaling and stop making Dpp whenever $hh^{ts}$ mutants are placed at the non-permissive temperature. Therefore, because cells in the developing *Drosophila* wing do not migrate or rearrange, the growth of the wings with *hh*⁻ clones indicates that Hh must travel across the clone of mutant cells from the more distant cells to signal the cells on the anterior side. The region of the wing near the compartment border is especially sensitive to levels of Hh and its growth is retarded when Hh signaling is reduced. Clones of *hh*⁻ cells that occupy >~25% of the posterior compartment cause a noticeable reduction in the size of this region (T Tabata, TB Kornberg, unpublished data), indicating a deficit in Hh signaling (Figure 1e; [6••]). Nevertheless, the remarkable capacity of these wings to suffer only subtle growth reductions in this region testifies to the long-range effectiveness of Hh.

Finally, Hh can signal to anterior cells far from the compartment border if anterior cells at the compartment border lose their ability to respond to Hh. *smo*⁻ cells do not transduce the Hh signal [20] and, as a consequence, do not elevate levels of Ptc as normal cells do. Ptc normally limits the range of Hh action, presumably by internalizing it (see above). Without Ptc, *smo*⁻ cells are an ineffective barrier and the Hh signal reaches cells farther into the anterior compartment. The behavior of large anterior *smo*⁻ clones at the compartment border is fascinating and particularly informative (Figure 1f; from [20]). The normal structures of the anterior compartment form, generated by the non-mutant anterior cells but, in addition, the clone of mutant cells produces a mirror symmetric anterior copy. As interpreted by Chen and Struhl [20], the compartment border remains at the juxtaposition between posterior cells and *smo*⁻ mutant anterior cells. The non-mutant anterior cells that respond to Hh produce Dpp and these cells, which are far from the compartment border, constitute the wing's developmental 'organizer'. In these wings, Hh-producing cells are far from the Hh-responding ones, revealing the long range over which Hh can act.

**Figure 2**




Schematic diagram of long-range effects of Shh in patterning the ventral neural tube and somite and the antero–posterior axis of the limb. **(a)** Transverse section through the spinal cord of developing mouse or chick embryos. Shh protein is synthesized by cells in the notochord (N) and floor plate (FP). V1 interneurons, V2 interneurons, motor neurons (MNs) and V3 interneurons are generated at distinct dorsal–ventral positions of the ventral neutral tube. These neurons can be induced in explants of naïve neural plate tissue exposed to Shh. The relative position at which each type of neuron is generated *in vivo* corresponds to the relative concentration of Shh required to induce that particular type of neuron *in vitro* with higher concentrations of Shh required to generate neurons occupying a more ventral position. Shh also induces scelerotomal (SC) fate in the ventral somite. **(b)** Dorsal view of the early chick limb bud showing localization of Shh protein to the zone of polarizing activity (ZPA). Different digit identities (2–4) are specified along the antero–posterior axis by signals from the ZPA. When Shh is expressed ectopically in the anterior margin of the limb bud, it is capable of inducing different digit identities in a dose-dependent manner. Higher concentrations of Shh induce digit identity of a more posterior character (such as digit 4) whereas lower concentrations of Shh induce digit identity of a more anterior character (such as digit 2). A, anterior; AER, apical ectodermal ridge; D, dorsal; DM, dermomyotome; FP, floor plate; N, notochord; P, posterior; RF, roof plate; SC, sclerotome; SE, surface ectoderm; V, ventral; ZPA, zone of polarizing activity.

In normal wings, the compartment border and developmental organizer coincide where cells that produce Hh abut responsive anterior cells (Figure 1a). In the wing illustrated in Figure 1f, these two cell populations are separated by a considerable distance, as are the compartment border and organizer. Numerous properties have been ascribed to compartment borders, namely straight boundaries of lineage restriction [21], separation of domains of gene expression [22], and reversal of developmental polarity [23]. The behavior of *smo*$^{-}$ mutant cells suggests that some of these are properties of the compartment border and others of the organizer. On the basis of the behavior of *smo*$^{-}$ clones [20,24,25], we propose that the organizer is responsible for the straightness of the border and for the reversal of developmental polarity, and that the lineage restriction and limits to domains of gene expression are properties of the compartment border.

## Long-range Hh signaling in vertebrates

With presently available antibody reagents and histological techniques, the Hh proteins have not been detected at sites other than the cells where they are expressed. Nevertheless, there is strong indirect evidence for long-range signaling in the ventral neural tube and somite as well as the antero-posterior axis of the limb in both mouse and chick. Shh protein synthesis is restricted to the notochord (which is underneath the ventral neural tube) and the floor plate (which is at the midline of the ventral neural tube) (Figure 2a; [26]). Shh appears to be able to induce ventral motor neurons and interneurons at specific dorsal–ventral positions of the neural tube. These ventral cell types can be induced in explants of naïve neural plate tissue after exposure to recombinant Shh protein [26–29]. Cell types that arise at a greater distance from the source of Shh *in vivo* are induced by lower concentrations of Shh in explants, and cell types that differentiate closer to the source of Shh are induced by concentrations of Shh that are only two–three-fold higher [27–29]. Similarly, dorsal–ventral patterning of somites, the pairs of mesoderm blocks that lie laterally to the neural tubes, is mediated by Shh in the notochord and floor plate (Figure 2a). Recombinant Shh protein induces ventral sclerotomal fate and represses dorsal dermomyotomal fate in presomitic mesoderm explants [30].

Finally, in the limb, Shh expression in the zone of polarizing activity — a small group of cells located at the distal posterior margin of the limb mesenchyme — signals to specify digit identity along the antero-posterior axis (Figure 2b; [31]). When implanted to the anterior margin of the chick limb, cells expressing different amounts of Shh induce digit duplication [32]. Moreover, higher concentrations of Shh induce digits of a more posterior character whereas lower concentrations of Shh induce digits of a more anterior character. These observations suggest that Shh can signal to cells several hundred microns away. This proposal is supported by an analysis of the limb buds of the chick *talpid*$^{3}$ mutant [33]. *Shh* expression appears to be normal in *talpid*$^{3}$ mutants but *Ptc* expression is reduced and the limbs have a polydactylous phenotype. These results raise the possibility that the polydactylous phenotype is a consequence of abnormal Shh signaling that

occurs when the lack of high-level Ptc expression allows Shh protein to acquire a wider distribution.

Additional evidence has been provided by studies of gene transcripts activated in response to Hh signaling, such as *Ptc-1* and *COUP-TFII*, which are detected at a distance from the Hh source [34,35]. Widespread *Ptc-1* expression can be detected in the posterior half of a developing mouse limb bud (Figure 2b), and expression of COUP-TFII, an orphan receptor in the steroid–thyroid hormone receptor superfamily, coincides with the appearance of motor neuron markers in the neural tubes (Figure 2a). Although there has been no demonstration that *Ptc-1* or *COUP-TFII* are direct transcriptional targets of Hh signaling, several observations support this possibility. First, consensus-binding sites for the Cubitus interruptus/Gli transcription factors are present in the Drosophila *ptc* promoter [36], and the Cubitus interruptus/Gli proteins are thought to be the principal mediators of Hh signal transduction. As almost every other aspect of the Hh pathway examined to date has been conserved between flies and vertebrates, this result implies that vertebrate *Ptc-1* may also be a direct transcriptional target of Hh signaling. Second, *COUP-TFII* expression is induced in response to Hh signaling in cultured cells and its activation in response to low-level Hh does not require new protein synthesis. If *Ptc-1* and *COUP-TFII* are direct targets of Hh, then their activation at a distance from the Hh source would argue that the long-range effect of Hh signaling is not mediated through a second signal. Perhaps the best evidence in support of a direct role for Shh is the recent finding that Gli3 protein processing is antagonized at long-range by Shh signaling [37]. As Gli3 processing is inhibited by Shh within a short period of time in culture and without a need for new protein synthesis, this effect of Shh signaling is likely to be direct.

## Conclusions: implications of long-range Hh signaling

The ability of Hh to signal at long range does not have a bearing on the likelihood that Hh also signals at short range, nor does it require the mechanisms that transport Hh from expressing to target cell to be the same over both short and long distances. Nevertheless, it seems reasonable to propose that the lipophilic character of Hh presents special problems for long-range signaling and that novel mechanisms would be needed. Four different modes of Hh distribution have been considered: diffusion that generates an extracellular gradient, serial transfers between neighboring cells ('bucket-brigade hand-offs') that generate an intracellular gradient, direct transfer at points of cytoneme contact [38], and secondary signals. It seems to be a truism that biological mechanisms optimize opportunities for intrinsic control and extrinsic regulation, and although we do not yet know which of these four mechanisms mediates Hh signaling, we can predict that the solution that has been devised is one that affords precisely controlled release and uptake of this powerful and ubiquitous morphogen.

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