Front. Biol. 2011, 6(6): 504–521
DOI 10.1007/s11515-011-1146-2

REVIEW

Hedgehog signaling: mechanisms and evolution

Xuan YE, Aimin LIU (✉)

Department of Biology, Eberly College of Science, The Pennsylvania State University, University Park, PA 16802, USA

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Abstract **The Hedgehog (Hh) family of secreted proteins plays essential roles in the development of a wide variety of animal species and underlies multiple human birth defects and cancers. To ensure the proper range of signaling, the Hh proteins are modified with lipids, assembled into water-soluble multimers, and interact with multiple cell surface proteins. In the target cells, a largely conserved intracellular signal transduction pathway, from the cell surface receptor Patched to the Glioma-associated oncogene homolog (Gli) family of transcription factors, mediates the transcriptional responses from fruit flies to mammals. A significant divergence between vertebrates and insects is the vertebrate-specific requirement of cilia for Hh signal transduction and Gli protein activation. Finally, transcription-independent cellular responses to Hh have been described in certain developmental processes. With clinical trial underway to treat Hh-related diseases, more work is urgently needed to reach a more comprehensive understanding of the molecular mechanisms underlying the regulation of Hh signaling in development and diseases.**

Keywords Hedgehog, Shh, Ihh, Dhh, Cubitus interruptus (Ci), Gli1, Gli2, Gli3, Patched, Smoothened, Cilia, *Drosophila*, mouse, signaling, development, evolution

Introduction

During the development and adult life of an animal, the proliferation, differentiation and migration of its cells need to be properly regulated and coordinated. Intercellular communication mediated by a few families of key signaling proteins plays crucial roles in regulating a variety of cell behaviors. Among them, the Hedgehog (Hh) family of proteins has received increasing attention due to its implication in multiple human birth defects and malignancies in various organ systems.

Hh was initially discovered as a "segment-polarity" gene that controls the cuticular patterns of the *Drosophila* larva (Nusslein-Volhard and Wieschaus, 1980) (Fig. 1A). Subsequent genetic studies in *Drosophila* indicated that Hh plays critical roles in the patterning of imaginal discs; primordia for adult organs such as the eyes and wings (Heberlein et al., 1993; Ma et al., 1993; Tabata and Kornberg, 1994). In the developing wing discs, the cells of the posterior compartment produce and secret Hh protein, which activates the expression of a plethora of target genes across the anterior/posterior (A/P) compartment border (Fig. 1B). One of these genes is *Decapentaplegic* (*Dpp*), which encodes a TGFβ-family protein that serves as a long-range signal to both compartments, regulating the formation of a normal wing.

The mammalian Hh family comprises three members: Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh). Among them, Shh received the most attention owing to its involvement in multiple key events in mammalian development. For example, *Shh* expression in the axial mesodermal structures, the notochord and prechordal plates, is critically important for proper patterning of the central nervous system (CNS) and somites along their dorsal/ventral (D/V) axes (Chiang et al., 1996) (Fig. 1C). In the developing spinal cord, high concentration of Shh induces the formation of floor plate, a group of glial cells that also expresses *Shh* (Roelink et al., 1995). Shh protein secreted from both the notochord and floor plate forms a ventral-to-dorsal gradient that defines the formation of various ventral interneurons and motor neurons in a stereotypical pattern. Loss of *Shh* leads to the loss of all ventral cell types of the spinal cord except for the ones normally located next to the D/V border (Chiang et al., 1996).

During limb development, *Shh* is expressed in a small group of mesenchymal cells near the posterior margin of the limb buds known as the zone of polarizing activity (ZPA) (Fig. 1D). Grafting ZPA or *Shh*-expressing cells to the anterior margin of the chicken limb buds induces a mirror-image duplication of the digits and posterior transformation of the skin, suggesting that Shh produced by ZPA cells

Received February 17, 2011; accepted March 11, 2011
Correspondence: Aimin LIU
E-mail: axl25@psu.edu

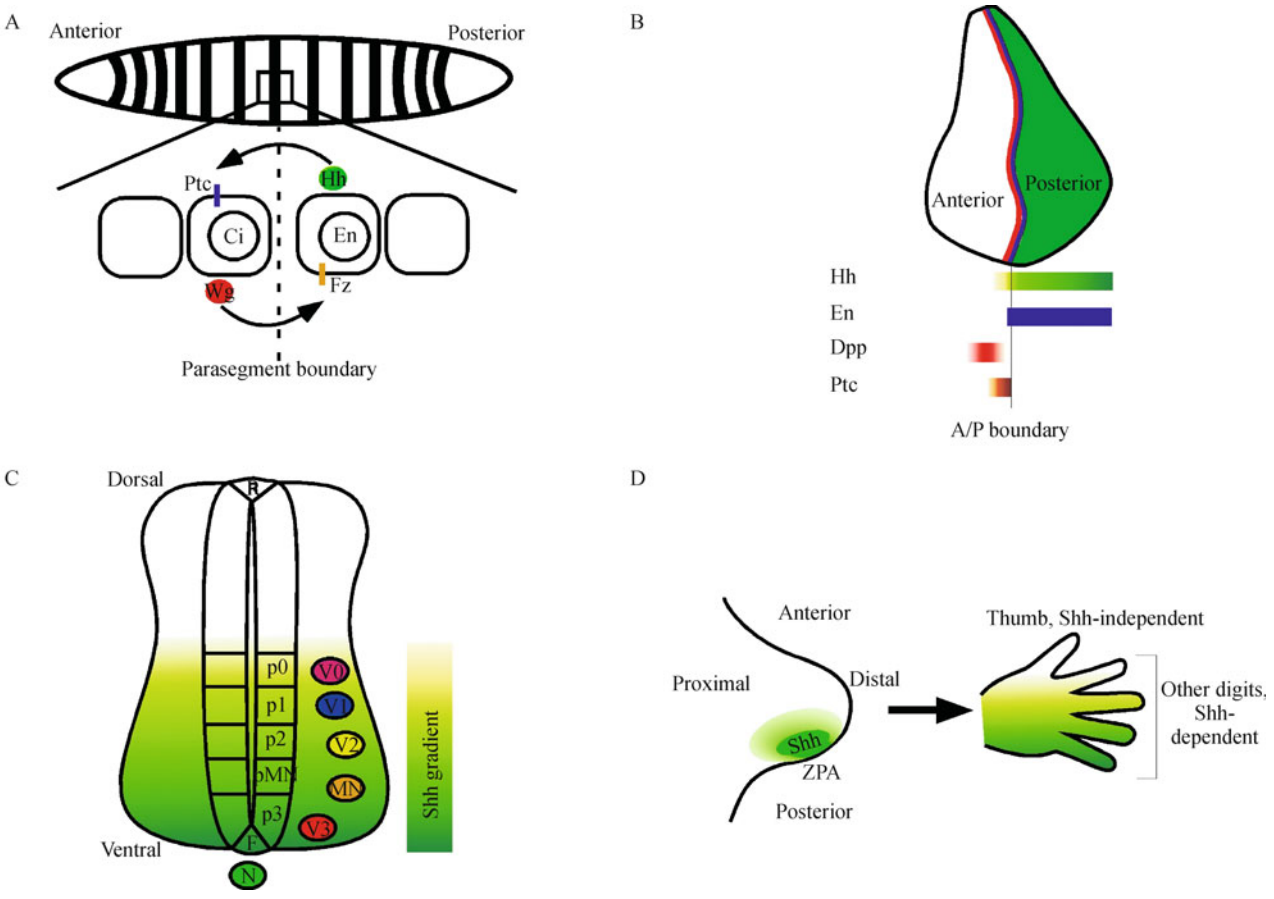


Figure 1 The Hedgehog family of proteins plays multiple roles in the development of *Drosophila* and vertebrates. (A) A *Drosophila* embryo are divided into 14 parasegments along its anterior/posterior axis. Cells immediately anterior to each parasegment boundary express a transcription factor Cubitus interruptus (Ci), a secreted signaling molecule Wingless (Wg) and a Hedgehog (Hh) receptor Patched (Ptc). Cells immediately posterior to each parasegment boundary express a transcription factor Engrailed (En), an Hh, and a Wg receptor Frizzled (Fz). Hh and Wg maintain each other's expression by activating Ci and En, respectively. This reciprocal signaling stabilizes the parasegment boundaries and ensures proper patterning in each parasegment. (B) Cells in the *Drosophila* wing imaginal discs, primordia for the fly wing, are segregated into anterior (A) and posterior (P) compartments. En maintains Hh expression in the P compartment, which in turn activates *En* expression in cells immediately anterior to the A/P compartment boundary. The expression of *Ptc* and *Decapentaplegic* (*Dpp*) is also induced in anterior cells near the compartment boundary. (C) In the vertebrate spinal cord, Shh produced by notochord (N) and floorplate (F) forms a ventral-to-dorsal concentration gradient. Neural progenitor cells in the spinal cord adopt various interneuron (p0, p1, p2 and p3) and motor neuron (pMN) fates depending on the level of Shh signaling they receive. These neural progenitors later differentiate into mature neurons (v0, v1, v2, MN and v3) in a stereotypical pattern. The formation of the floorplate itself is dependent on the highest level of Shh activity. R: roofplate. (D) In vertebrate limb buds, Shh produced in the zone of polarizing activity (ZPA) is critical for patterning the digits along the anterior/posterior axis. The first digit in each limb (thumb or big toe) is the only digit that is independent of Shh.

provides the positional information for proper limb patterning along the A/P axis (Krauss et al., 1993). A complete loss of *Shh* in mouse leads to a great reduction of digits, suggesting an additional role of Shh in the survival or proliferation of the limb cells (Chiang et al., 2001; Kraus et al., 2001).

The other two mammalian Hh family members play more tissue-specific roles. Ihh plays a prominent role in bone formation (St-Jacques et al., 1999). It is also involved in vascular development in the yolk sacs (Dyer et al., 2001; Byrd et al., 2002). *Dhh* is expressed in the Sertoli cells of the testes and is required for spermatogenesis (Bitgood et al., 1996).

Because Hh proteins are critically important in development, the availability of the ligands and the competence to respond to the ligands by the target cells are under stringent regulation in both vertebrate and invertebrate animals. Interestingly, although largely conserved through evolution, some aspects of the Hh signaling pathway are amazingly divergent between fruit flies and vertebrates. One important difference between the vertebrate and *Drosophila* Hh pathways is the vertebrate-specific requirement for cilia. In this review, we will focus on the molecular mechanisms underlying the production, distribution and reception of Hh signals and highlight the similarity, as well as the divergence, between the Hh signaling pathways in *Drosophila* and vertebrates. To help the readers better understand the content of this article, we summarized the names and roles of the key

regulators of Hh signaling in *Drosophila* and vertebrates in Table 1.

Production and distribution of Hh

Production and distribution of Hh in *Drosophila*

The *Drosophila* Hh gene encodes a 45 kDa protein that undergoes autoproteolytic processing (Lee et al., 1994) (Fig. 2). The N-terminal product of this processing, denoted as Hh-Np, exhibits all signaling activities of Hh and carries a cholesterol moiety to its C terminus (Porter et al., 1995; Porter et al., 1996a, 1996b). The autoproteolysis of Hh and cholesterol modification of Hh-Np is dependent on the C-terminal half of the protein because Hh-N, a truncated form of Hh encoded by a transgene mimicking the processed product, is not modified by cholesterol. The cholesterol moiety appears to play important roles in restricting the spread of Hh and increasing its local concentration because Hh-N travels a much longer distance than Hh-Np in tissues (Porter et al., 1996a; Dawber et al., 2005; Callejo et al., 2006).

The mature, secreted form of Hh, Hh-Np, also contains a palmitate moiety attached to a Cysteine residue near its N terminus (Chamoun et al., 2001) (Fig. 2). The palmitoylation of Hh is critical for its function, as mutations in an apparent transmembrane acyltransferase, Skinny Hedgehog (Ski; also known as Sightless, Central missing or Rasp), which presumably catalyzes the palmitoylation of Hh-N, leading to developmental defects suggestive of compromised Hh signaling activities (Amanai and Jiang, 2001; Chamoun et al., 2001; Lee and Treisman, 2001; Micchelli et al., 2002). In addition, Hh protein carrying a cysteine-to-alanine mutation at the palmitoylation site exhibits greatly reduced signaling activity, further confirming the importance of palmitoylation in the signaling activity of Hh protein.

The dual lipid modification of Hh makes it highly hydrophobic and likely prevents its free release from Hh-producing cells. Dispatched (Disp), a 12-pass transmembrane protein and a member of the resistance-nodulation division (RND) family of proton-driven transporters, is required for the release of Hh-Np, but not Hh-N (Burke et al., 1999) (Fig. 2). In the absence of Disp, Hh protein is retained in cells where it is produced and fails to activate its target cells.

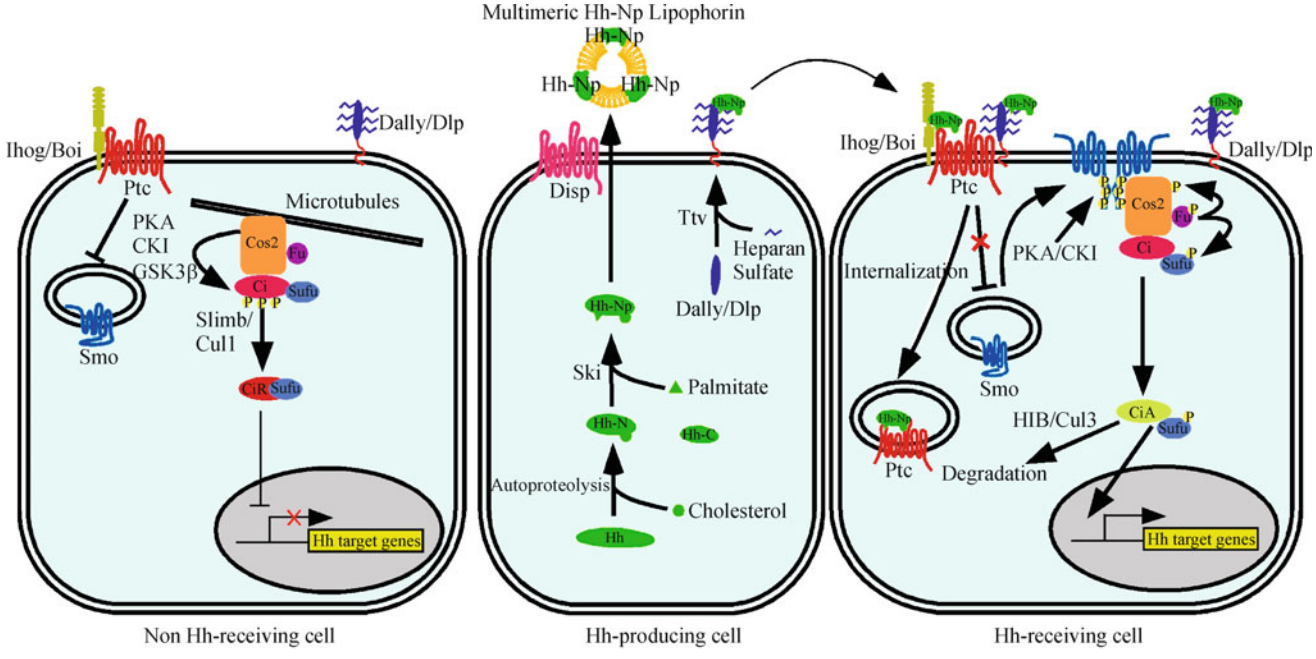


Figure 2 Hedgehog signaling pathway in *Drosophila*. In the absence of Hedgehog (Hh), Patched (Ptc) inhibits the activity and cell surface localization of Smoothened (Smo). The transcription factor Ci forms a complex with Cos2, Fu and Sufu. Cos2 promotes the phosphorylation and subsequent Slimb/Cul1-mediated proteolytic processing of Ci by recruiting PKA, CKI and GSK3β. The processed Ci (CiR) enters the nucleus to repress the Hh target genes.

In Hh-producing cells, the Hh protein undergoes autoproteolysis during which the N-terminal half of the protein (Hh-N) is modified with cholesterol. Hh-N is palmitoylated through the function of an acyltransferase Skinny Hedgehog (Ski). The fully processed Hh (Hh-Np) is released into extracellular space through the function of Dispatched (Disp). The spread of Hh-Np is aided by the formation of a multimeric complex with lipophorin and interaction with GPI-linked heparan sulfate proteoglycans Dally and Dally-like protein (Dlp). Tout-velu (Ttv), a glycosyltransferase catalyzing the maturation of Dally/Dlp, is critical for long-range Hh signaling.

In Hh-receiving cells, Ihog/Boi and Dlp interact with Hh and facilitate the inactivation of Ptc by Hh. Smo is translocated to the cell surface and activates Ci through direct interaction with Cos2. The activation of Smo involves PKA and CKI-mediated phosphorylation of Smo, as well as the phosphorylation of Cos2, Fu and Sufu. Activated Ci (CiA) enters the nucleus, possibly together with Sufu, to activate Hh target genes. CiA is highly unstable owing to HIB/Cul3-mediated proteasomal degradation.

Table 1 The Hh pathway components and their roles in fruit flies and vertebrates

Drosophila	Vertebrates	Roles in Hh signaling
Hedgehog (Hh)	Sonic, Indian and Desert hedgehog (Shh, Ihh and Dhh)	Ligands
Skinny hedgehog (Ski)	Skinny hedgehog (Skn)	Transmembrane acyltransferase critical for the palmitoylation of Hh proteins.
Dispatched (Disp)	Dispatched1 (Disp1)	A 12-pass transmembrane protein mediating the release of Hh-Np from Hh-producing cells
Apolipophorins (ApoLI and ApoLII)	Low density lipoprotein (LDL)	Form lipoprotein particles with Hh-Np and facilitate the long-range distribution of Hh in *Drosophila*. Roles unclear in vertebrates
Megalin	Megalin	An LDL receptor-related protein interacting with Shh in vertebrates, but not in *Drosophila*
Dally and Dally-like protein (Dlp)	Glypicans (Glp1-6)	GPI-linked heparan sulfate proteoglycans required for long-range Hh signaling and Hh reception in *Drosophila*. In vertebrates, they appear to limit the range of Hh signaling.
Tout-velu (Ttv)	Exostosin 1 (Ext1)	A glycosyltransferase required for glypican maturation. Facilitate long-range Hh signaling in *Drosophila*, but limit long-range Hh signaling in vertebrates.
Smoothened (Smo)	Smoothened (Smo)	A seven-pass transmembrane protein structurally related to G protein-coupled receptors, essential for Hh signal transduction
Patched (Ptc)	Patched (Ptch1 and 2)	12-pass transmembrane proteins and the receptors for Hh proteins.
Interfering hedgehog (Ihog)/Brother of Ihog (Boi)	Cdo/Brother of Cdo (Boc)	Immunoglobulin/fibronectin superfamily of transmembrane proteins interacting with Hh proteins and playing important roles in Hh reception and distribution.
————	Growth arrest specific 1 (Gas1)	A GPI-linked Hh-interacting protein with a positive role in mammalian Hh signaling. No *Drosophila* homolog identified
————	Hedgehog interacting protein (Hhip1)	A Hh-interacting transmembrane protein with a negative role in Hh signaling. No *Drosophila* homolog identified
Cubitus interruptus (Ci)	Glioma-associated homologs (Gli1, 2, 3)	Bipartite transcription factors (except for Gli1) mediating all transcriptional responses of Hh signaling.
Costal 2 (Cos2)	Kif7 and Kif27	Kinesin-like proteins. Both Cos2 and Kif7 are involved in Hh signaling and physically interact with Smo and Ci/Gli proteins. Kif27 is involved in motile cilia biogenesis
Fused (Fu)	Fused (Fu)	A serine/threonine kinase, part of a Ci-containing complex important for Hh signaling in *Drosophila*. In vertebrates, it is important for motile cilia biogenesis
Suppressor of fused (Sufu)	Suppressor of fused (Sufu)	A cytoplasmic protein directly interacting with Ci/Gli proteins. An essential negative regulator of Hh signaling in vertebrates, but dispensable in *Drosophila*.
Hedgehog-induced Math and BTB domain-containing protein (Hib)	Speckle type POZ protein (Spop)	Part of a ubiquitin ligase complex involved in the proteasomal degradation of Ci/Gli proteins
Supernumerary limbs (Slimb)	β-Transducin repeat containing protein (β-TrCP)	Part of a ubiquitin ligase complex involved in the proteolytic processing of Ci/Gli proteins
cAMP-dependent protein kinase (PKA)/casein kinase I (CKI)	cAMP-dependent protein kinase (PKA)/Casein Kinase I (CKI)	Serine-threonine kinases involved in the activation of Smo and proteolytic processing of Ci/Gli protein in both *Drosophila* and vertebrates
Glycogen synthase kinase 3 (Gsk3)	Glycogen synthase kinase 3 (Gsk3)	A serine-threonine kinase involved in the proteolytic processing of Ci/Gli protein in both *Drosophila* and vertebrates
Gαi	Gαi	A subunit of a trimeric G protein complex involved in Hh signaling downstream of Smo in *Drosophila*. Its involvement in vertebrates is unclear
————	Protein phosphatases (PP4 and PP2a)	PP4 mediates the dephosphorylation of Smo and PP2a mediates the dephosphorylation of Gli proteins
————	Pias1	An E3 ubiquitin ligase mediating SUMOylation of Gli proteins
————	HDAC1	A deacetylase mediating the deacetylation of Gli1 and Gli2

The lipid modification of Hh-Np also suggests that Hh proteins tend to attach to the cell membrane and cannot travel through tissue efficiently by diffusion. However, Hh-Np does travel through the wing disc tissue to activate the expression of a range of target genes (Fig. 1B). One study showed that Hh-Np is mobilized through the association with apolipophorins, the *Drosophila* equivalent of low-density lipoprotein (LDL) (Panakova et al., 2005) (Fig. 2). Long-range Hh signaling is disrupted when the formation of this Hh-Np-containing lipoprotein complex is disrupted. Interestingly, Megalin, an LDL receptor-related protein, interacts with Shh in vertebrates (McCarthy et al., 2002). However, there is no evidence that the Megalin homolog or other LDL receptor-related proteins are associated with Hh signaling in *Drosophila*.

The distribution of Hh-Np is also regulated by GPI-linked cell surface glycoproteins Dally and Dally-like protein (Dlp) (Desbordes and Sanson, 2003; Lum et al., 2003a; Han et al., 2004) (Fig. 2). The heparan sulfate chains on these two proteins are required for their function in facilitating the spread of Hh proteins. Tout-velu (Ttv), a glycosyltransferase required for the maturation of heparan sulfate proteoglycans like Dally and Dlp, is essential for the efficient long-range distribution of Hh protein in *Drosophila* tissues (Bellaiche et al., 1998; The et al., 1999).

Production and distribution of Hh in vertebrates

Vertebrate Shh proteins undergo proteolytic processing and cholesterol modification similar to their *Drosophila* homolog (Lee et al., 1994; Bumcrot et al., 1995; Porter et al., 1995; Porter et al., 1996b). There were contradictory reports regarding the effect of cholesterol modification on the signaling range of Shh in mammals. An earlier report stated that replacing endogenous Shh with Shh-N by introducing a nonsense mutation disrupted long-range, but not short-range Shh signaling (Lewis et al., 2001). This result appears to suggest that cholesterol modification of Shh is required for its long-range distribution in the tissues, which is inconsistent with what was observed in *Drosophila*. However, a decrease in the *Shh* mRNA level in the mutant embryos, likely due to nonsense-mediated decay, may be the real underlying cause of the defects observed in their study. In a more recent study, Shh-Np was replaced with Shh-N without changing the *Shh* mRNA level (Li et al., 2006). These *Shh-N* expressing mouse embryos form extra digits in their limbs, suggesting that cholesterol modification limits the range of Shh signaling in mammals.

In *in vitro* studies, Shh-Np, the fully processed form, is associated with the cell membrane, whereas Shh-N, which lacks the cholesterol moiety, is freely diffusible (Zeng et al., 2001). However, a small amount of Shh-Np does exist in a soluble multimeric form, rather than in a multimeric Shh-Np complex (Fig. 3). It is likely that Shh-Np self-associates and forms a globular multimeric protein complex with its hydrophobic lipid-moiety buried inside, hence significantly increasing its solubility. It is not clear whether lipoproteins play significant roles in the assembly of the Shh multimeric complex and long-range signaling in vertebrates. However, Megalin interacts with Shh, suggesting that lipoproteins may be involved in Hh signaling in vertebrates as well (McCarthy et al., 2002).

Similar to *Drosophila* Hh, vertebrate Shh proteins are palmitoylated (Pepinsky et al., 1998) (Fig. 3). Palmitoylation is critical for Hh signaling and the normal patterning of the spinal cord and limbs (Chen et al., 2004). Without palmitoylation, mouse Shh protein fails to assemble a multimeric complex that exhibits much more potent signaling activity and higher mobility in tissues than Shh monomers.

The essential roles of Dispatched in Hh signaling are conserved in mammals (Fig. 3). In the absence of mouse Dispatched 1 (Disp1), Hh signaling and embryonic development is severely disrupted (Caspary et al., 2002; Kawakami et al., 2002; Ma et al., 2002). Consistent with a role in releasing lipid-modified Hh proteins, removing *Disp1* specifically from *Shh*-expressing cells elicits similar developmental defects as those in which *Disp1* is removed in the entire embryo (Tian et al., 2005). Moreover, the signaling activity of Shh-N, which is not modified with cholesterol, is not dependent on Disp1 (Tian et al., 2005; Li et al., 2006).

Finally, the roles of GPI-linked heparan sulfate proteoglycans in the distribution of Hh ligands do not appear to be conserved between *Drosophila* and vertebrates. Exostosin 1 (Ext1), one of the mammalian homologs of Ttv, appears to limit, instead of promote, the range of Ihh signaling in bone development (Koziel et al., 2004). Glypican-3, one of the six mouse glypican proteins, also appears to negatively regulate Shh activity (Capurro et al., 2008). More research into the molecular mechanisms underlying the interactions between various Hh family proteins and proteoglycans in different species is apparently needed to better understand this divergence.

Hh-dependent Smo activation

Hh-dependent Smo activation in *Drosophila*

Smoothened (Smo), a seven-pass transmembrane protein structurally related to G protein-coupled receptors, plays a central role in mediating the target cell responses to Hh (Alcedo et al., 1996). In the absence of Hh, the majority of Smo is present in endosomal compartments, whereas Hh stimulates the translocation of Smo to the cell surface (Denef et al., 2000) (Fig. 2). The cAMP-dependent protein kinase (PKA) and Casein kinase I (CKI) promote the cell surface localization of Smo through direct phosphorylation of the intracellular C-terminal tail of Smo (Jia et al., 2004; Zhang et al., 2004; Apionishev et al., 2005). Cell surface localization

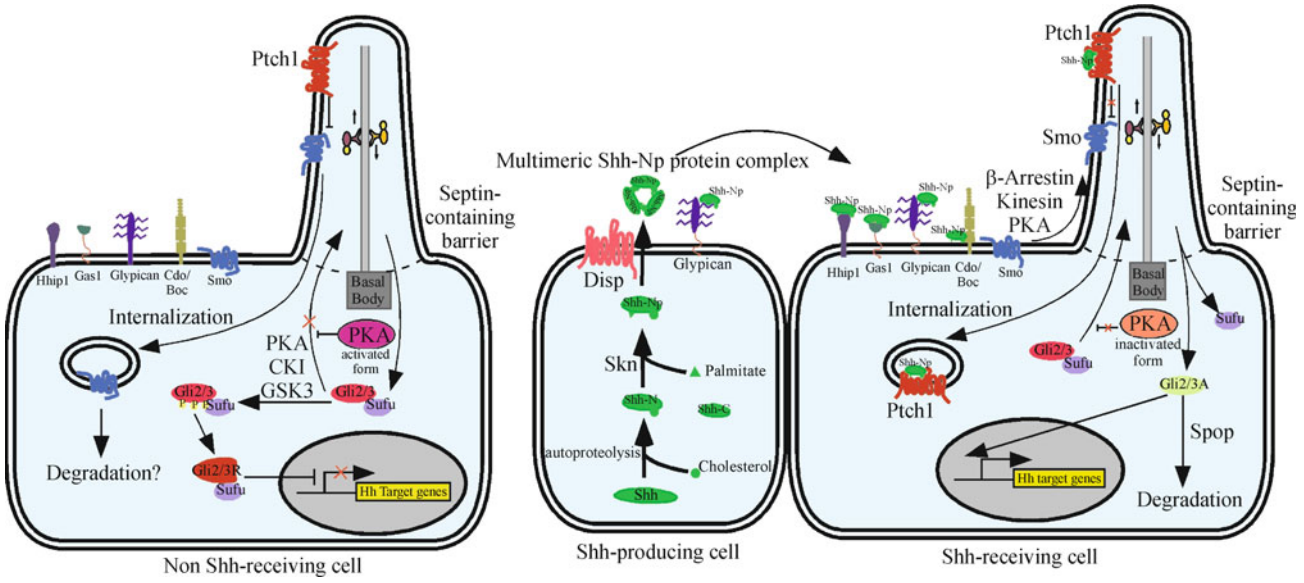


Figure 3 Sonic hedgehog signaling pathway in vertebrates. In the absence of Sonic hedgehog (Shh), Ptc1, but not Smo, is accumulated in the ciliary membrane. Gli2 and Gli3 proteins (Gli2/3), associated with Sufu, are phosphorylated by PKA/CKI/GSK3 and proteolytically processed into Gli2/3R. Gli2/3 and Sufu are not efficiently translocated to the cilia, possibly due to high PKA activity in the cell.

In Shh-producing cells, Shh undergoes autoproteolysis, cholesterol and palmitate dual modification to form processed Shh-Np. Disp is required for the release of Shh-Np, which is assembled into a multimeric protein complex. Glypicans homologous to Dally/Dlp interact with Shh (as well as Ihh), and limit the range of Shh signaling.

In Shh-receiving cells, Shh binds to Ptch1 and induces its internalization. Other cell surface proteins, such as Hhip1, Gas1, Glypican, Cdo/Boc (vertebrate homologs of Ihog/Boi), also interact with Shh. However, their localization and relationships with Ptch1 have not been investigated. Smo responds to Shh by trafficking into the cilia, a process facilitated by β-Arrestin, Kinesin and PKA. Shh also enhances the ciliary accumulation of Gli2/3 and Sufu, possibly by inhibiting PKA activity. Gli2/3 are activated in the cilia to form Gli2/3A and likely enter the nucleus without Sufu. Gli2/3A are unstable and subject to Spop/Cul3-mediated degradation.

appears to be important for the activation of Smo, because forcing Smo to the cell surface activates Hh signaling, whereas sequestering it in the endoplasmic reticulum inhibits Hh signaling (Zhu et al., 2003).

Like other G protein-coupled receptors, the activation of Smo involves a change in conformation (Zhao et al., 2007) (Fig. 2). In the absence of Hh signaling, intra-molecular interactions between the C-terminal tail and one of the intracellular loops of Smo prevents its interaction with other proteins. Graded Hh pathway activation likely increases the phosphorylation of the C-terminal region of Smo, disrupting intra-molecular interaction and allowing the interactions between C-tails of dimerized Smo and downstream components of the signaling pathway. This model provides at least part of the answers to how target cells respond to different levels of Hh.

Although Smo is structurally related to G protein-coupled receptors, there has been no evidence that Smo binds Hh or any other ligands. Instead, genetic studies indicated that the Hh signal is transmitted to Smo through Patched (Ptc), a member of the RND proton-driven transmembrane transporter family highly similar to Disp (Hooper and Scott, 1989; Nakano et al., 1989; Alcedo et al., 1996) (Fig. 2). Ptc negatively regulates the activity of Smo, but the molecular mechanism by which it does so remains elusive (Alcedo et al., 1996). It is unlikely that Ptc inhibits Smo through direct physical interaction. First, protein localization studies suggested that Ptc and Smo are localized to different compartments in the cells (Denef et al., 2000; Zhu et al., 2003). Furthermore, a small amount of Ptc is sufficient to suppress the activity of Smo, suggesting that the catalytic activity of Ptc, rather than its direct physical interaction with Smo, underlies the repression of Smo activity (Taipale et al., 2002).

The discoveries of small lipophilic molecules that directly activate or inhibit *Drosophila* and/or vertebrate Smo activity suggest that Ptc may inhibit Smo through regulating the availability of an endogenous Smo agonist or antagonist (Chen et al., 2002a; Chen et al., 2002b; Frank-Kamenetsky et al., 2002). However, the identity of such an endogenous factor has not been revealed. A recent study suggested that Ptc represses Smo accumulation on cell surface and directs Smo to a degradation pathway by increasing lipoprotein-derived lipid composition of the endosomal compartment (Khaliullina et al., 2009). In the presence of Hh, Ptc is sorted to degradation from endosomes, and Smo is promoted to the recycle pathway to accumulate on the cell surface. Another potential mechanism by which Ptc regulates Smo is through its role in biosynthesis of phosphoinositol 4-phosphate (PI4P) (Yavari et al., 2010). It is possible that Ptc regulates Smo through the combination of multiple mechanisms.

Finally, although *Drosophila* Ptc has long been widely considered as the Hh receptor, its physical interaction with Hh

has only been demonstrated recently (Zheng et al., 2010). Two members of the Immunoglobulin/fibronectin superfamily of transmembrane proteins, Interference hedgehog (Ihog), and Brother of Ihog (Boi), directly interact with Hh and are required for the cell surface presentation of Ptc and efficient interaction between Ptc and Hh (Yao et al., 2006; Zheng et al., 2010) (Fig. 2). A new definition of the Hh receptor was hence proposed to include both Ihog/Boi and Ptc. However, genetic analyses indicated that Ihog/Boi plays both positive and negative roles in Hh signaling, suggesting that more characterization of these proteins is still warranted (Yan et al., 2010).

Interestingly, in addition to the non-cell-autonomous roles in Hh protein distribution, Dlp also exhibits an essential cell-autonomous function in Hh target cell response (Williams et al., 2010; Yan et al., 2010). This cell-autonomous role is independent of the heparan sulfate modification of the proteins. A study using wing imaginal discs showed that Dlp physically interacts with both Ptc and Hh and promotes cell surface localization of Ptc, but another group failed to confirm the interaction in an *in vitro* binding assay (Williams et al., 2010; Yan et al., 2010).

Finally, in addition to regulating Smo activity, Ptc, Ihog and Boi also play a role in Hh distribution (Chen and Struhl, 1996; Zheng et al., 2010). The association of Hh with Ptc leads to the internalization of both proteins, limiting the range of Hh signaling. Reducing the level of Ihog allows longer range Hh signaling even in the presence of Ptc, consistent with its role in presenting Ptc to Hh. However, it is not clear whether Ihog is internalized with Ptc and Hh.

Hh signal transduction in vertebrates requires primary cilia

Cilia are microtubule-based cell surface organelles that originate from a centrosome-like cytoplasmic structure named the basal body (Gerdes et al., 2009) (Fig. 4). Based on their structure and motility, cilia can be categorized into three groups. All cilia contain a microtubule "backbone" called an axoneme, which comprises nine microtubule doublets arranged in a cartwheel pattern. Some epithelial cells lining the airways, reproductive tracts and cerebral ventricles have numerous motile cilia, whose axonemes contain a pair of microtubules in the center (hence the name 9 + 2 cilia). The coordinated beating movement of these cilia is critical for transporting fluids and/or cells. Most other vertebrate cells contain a single non-motile cilium devoid of the central pair of microtubules (9 + 0 cilium), the primary cilium. Node cilia, primary cilia present on the ventral surface of the embryonic node, are the only known motile primary cilia. The gyrating movement of the node cilia (and their equivalents in other vertebrates) appears to underlie proper patterning along the left-right axis in vertebrates (Hirokawa et al., 2006). In humans, cilia dysfunction and malformation accounts for birth defects in multiple organ systems, including retinal dystrophy, polydactyly, renal malformation, neural tube defects, hydrocephalus and mental retardation. Furthermore, lack of cilia motion in the respiratory tract results in respiratory deficiency.

The biogenesis of all cilia, as well as the structurally related eukaryotic flagella, shares the same molecular mechanism. Studies on flagella of the single cell green alga, *Chlamydomonas reinhardtii*, have revealed the functional importance of two multi-protein complexes, intraflagellar transport particles (IFT-A and IFT-B) (Rosenbaum and Witman, 2002) (Fig. 4). IFT-B, along with the trimeric anterograde motor kinesin II, appears to be responsible for trafficking cargoes from the base to the tip of the cilium. On the other hand, IFT-A, associated with retrograde motor cytoplasmic dynein, appears to carry cargoes back to the base of the cilium. Mutations in IFT-B proteins or Kinesin subunits result in degeneration of flagella in algae and loss of cilia in mice, indicating that IFT is required for flagella/cilia formation (Gerdes et al., 2009). Mutations in IFT-A components or dynein subunits lead to enlarged tips of the cilia/flagella, consistent with their roles in retrograde transport.

The ciliary membrane that envelops the axoneme is an extension of the plasma membrane, but with distinct lipid and protein composition (Rohatgi and Snell, 2010) (Fig. 4). The biogenesis of the ciliary membrane, as well as the ciliary translocation of specific transmembrane proteins, is dependent on the BBSome, a multiprotein complex comprising seven Bardet-Biedl Syndrome-related proteins (Nachury et al., 2007; Jin et al., 2010).

In 2003, we reported that Hh signaling is disrupted in three mouse mutants with defective intraflagellar transport (Huangfu et al., 2003). Mutations in these genes, namely *Ift88, Ift172* and *Kif3a*, lead to diminished expression of Hh target genes, as well as abnormal patterning of the ventral CNS, suggesting an important role for IFT in Hh signaling. We subsequently showed that cells with defective IFT are no longer responsive to Shh, substantiating an essential role of IFT in mediating target cell responses to Hh ligands (Liu et al., 2005).

In addition to defects in Hh signaling, the complete loss of *Ift88, Ift172* and *Kif3a* disrupts ciliogenesis, implicating a connection between cilia and Hh signaling. This connection is substantiated by subsequent studies of more mouse mutants with defective cilia formation and Hh signaling (Huangfu and Anderson, 2005; Liu et al., 2005; May et al., 2005; Houde et al., 2006; Caspary et al., 2007; Hoover et al., 2008; Heydeck et al., 2009; Zeng et al., 2010a; and more).

The requirement of cilia in Hh signaling appears to be ubiquitous in vertebrates. Cilia defects in chicken, frogs and zebrafish all lead to the disruption of Hh signaling (Corbit et al., 2005; Park et al., 2006; Yin et al., 2009). In contrast, disruption of cilia formation in *Drosophila* does not have any impact on Hh signaling, suggesting a major divergence in the molecular mechanisms underlying the intracellular transduction of Hh signals (Han et al., 2003; Sarpal et al., 2003).

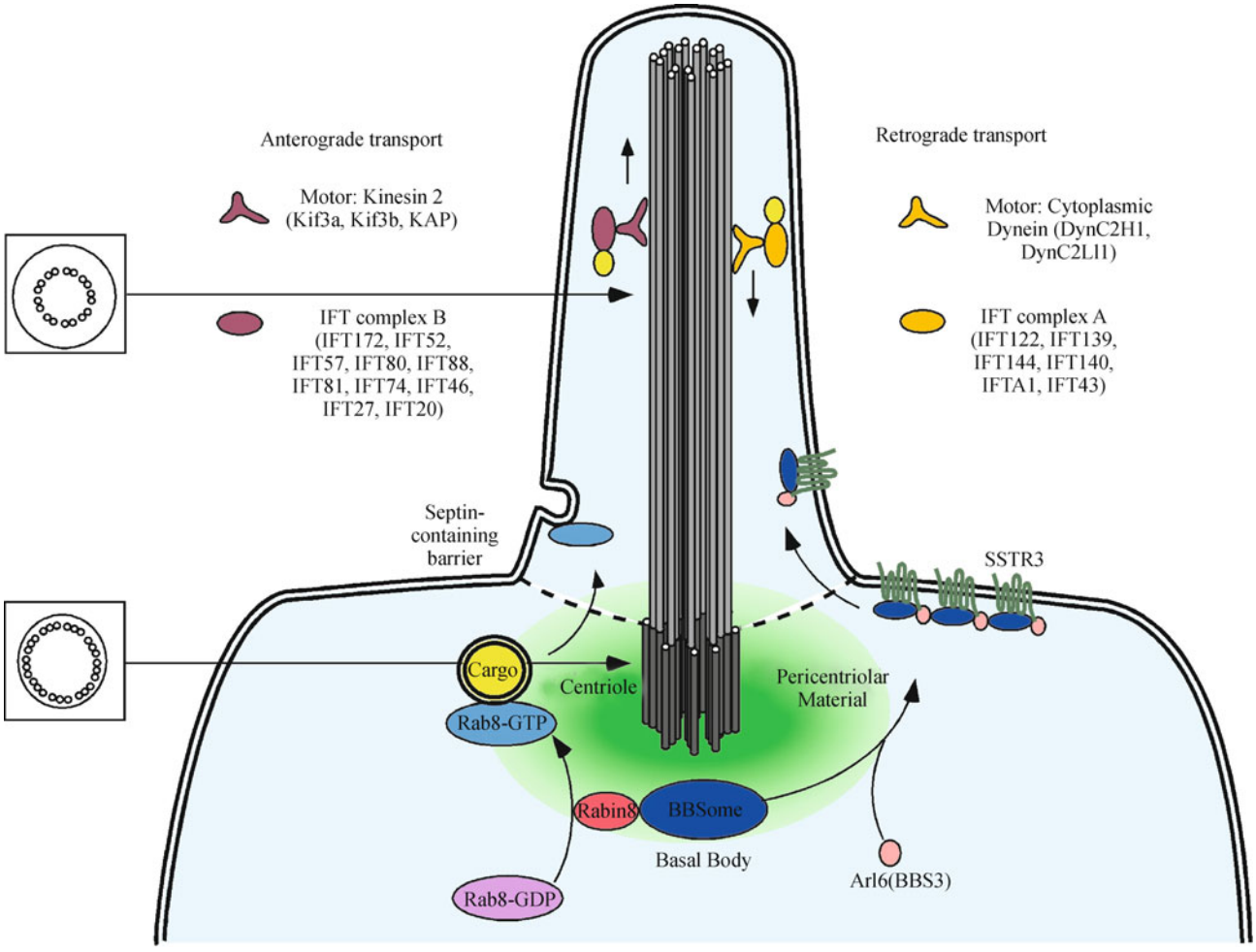


Figure 4 The primary cilium.The primary cilium is present on almost every vertebrate cell. It consists of an axoneme enclosed in the ciliary membrane. The axoneme, with nine microtubule doublets arranged in a cartwheel pattern, grows out of a centriole at the base of the cilium. The centriole, comprising nine microtubule triplets, and proteins associated with it, form the basal body of the cilium. Intraflagellar transport (IFT) complex B and the trimeric kinesin 2 constitute the anterograde IFT system responsible for cargo trafficking to the top of the cilium. On the other hand, IFT complex A and cytoplasmic dynein carry out retrograde IFT, returning cargoes to the base of the cilium. BBSome, a multiprotein complex comprising seven Bardet-Biedl syndrome-related proteins, is localized to the pericentriolar material and the cilium. BBSome binds Rabin8, a Rab8-specific GDP/GTP exchange factor, and promotes Rab8 activation and ciliary membrane biogenesis. In addition, BBSome is recruited by Arl6 (BBS3)-GTP to form a membrane coat that targets certain transmembrane proteins (such as SSTR3) to the cilium. Septin located at the base of the ciliary membrane forms a barrier that is important for the separation between ciliary membrane and the rest of the plasma membrane.

Hh-dependent Smo activation in vertebrates

Similar to *Drosophila*, the activation of mammalian Smo appears to require both changes in its subcellular localization and conformation (Corbit et al., 2005; Zhao et al., 2007) (Fig. 3). In the absence of Hh ligands, Smo is localized to the plasma membrane and intracellular vesicles (Milenkovic et al., 2009). The addition of Shh triggers the translocation of Smo to the cilia within one hour (Corbit et al., 2005; Milenkovic et al., 2009; Rohatgi et al., 2009; Wang et al., 2009; Wilson et al., 2009a). The ciliary translocation of Smo is dependent on β-arrestin 1 and 2, which appears to mediate the interaction between Smo and the anterograde IFT motor kinesin (Kovacs et al., 2008). The efficient enrichment of Smo in the ciliary membrane is also maintained by a Septin-containing barrier that prevents the free diffusion of membrane content between ciliary membrane and the rest of the plasma membrane (Hu et al., 2010).

The ciliary translocation of Smo appears to be an important part of Smo activation. A mutant form of Smo that fails to enter cilia cannot be activated, whereas SAG, a small molecule agonist of Smo, triggers ciliary translocation of Smo (Corbit et al., 2005; Rohatgi et al., 2007). PKA activation promotes Smo ciliary localization, consistent with its positive role in Smo activation (Milenkovic et al., 2009; Wilson et al., 2009a). However, ciliary accumulation of Smo is not sufficient for its activation. Cyclopamine, a potent antagonist of Smo, causes accumulation of inactive Smo in

the cilia (Rohatgi et al., 2009; Wang et al., 2009; Wilson et al., 2009a). Furthermore, disruption of dynein-dependent retrograde transport leads to the ciliary localization of Smo, but not the activation of Hh pathway (Huangfu and Anderson, 2005; Ocbina and Anderson, 2008). Because a conformational change is required for the activation of Smo in vertebrates, it is possible that cyclopamine treatment and loss of dynein lead to the ciliary accumulation of Smo locked in its inactive conformation (Zhao et al., 2007).

There are two vertebrate homologs of Patched, Ptch1 and Ptch2, but only Ptch1 plays an essential role in regulating Smo function (Goodrich et al., 1997; Lee et al., 2006) (Fig. 3). In mammals, loss of one copy of *Ptch1* leads to frequent formation of skin and brain tumors, whereas loss of both copies of *Ptch1* results in ubiquitous activation of Hh signaling in the embryos. Ptch1 is also localized to the cilia, but only when Hh ligands are absent (Rohatgi et al., 2007). Addition of Shh leads to the exclusion of Ptch1 from the cilia. Interestingly, loss of Ptch1 leads to the constitutive localization of Smo to the cilia, suggesting that Ptch1 regulates the ciliary localization of Smo.

Cdo and brother of Cdo (Boc), mammalian homologs of Ihog and Boi, bind the Shh ligand and play positive roles in Hh signaling (Okada et al., 2006; Tenzen et al., 2006; Bergeron et al., 2011) (Fig. 3). However, structural analyses suggest that the protein domains involved in the Cdo/Shh interaction are different from those involved in the Ihog/Hh interaction, suggesting against simple evolutionary conservation (McLellan et al., 2008). In addition, overexpressed Ptch1 can directly interact with Shh without co-expressed vertebrate Cdo or Boc, which is different from what was reported in *Drosophila* (Marigo et al., 1996; Stone et al., 1996).

Growth arrest-specific gene 1 (*Gas1*), which encodes an N-glycosylated GPI-linked membrane protein, plays a positive role in mammalian Hh signaling (Allen et al., 2007; Martinelli and Fan, 2007) (Fig. 3). The Hh interacting protein (Hhip) also interacts with Shh on the surface of the target cells and plays a negative role in Hh signaling (Chuang and McMahon, 1999; Chuang et al., 2003). Interestingly, both Gas1 and Hhip appear to lack a *Drosophila* homolog, indicating additional divergence between Hh signaling in vertebrates and invertebrates.

Signal transduction from Smo to Gli proteins

Signal transduction from Smo to Cubitus interruptus in *Drosophila*

What does Smo do upon its activation? The simple answer to this question is that it changes the activities of a zinc-finger transcription factor, Cubitus interruptus (Ci). However, what constitutes these changes and how Smo activation leads to these changes remains incompletely understood and is under active investigation.

Ci is a dual-functional transcription factor with both a repressor domain at its N terminus and an activator domain at its C terminus (Orenic et al., 1990). In the absence of Hh, Ci is extensively phosphorylated by PKA, CKI and glycogen synthase kinase 3 (GSK3) (Chen et al., 1998; Price and Kalderon, 1999; Price and Kalderon, 2002) (Fig. 2). The phosphorylated Ci is recognized by Slimb/Cul1-containing E3 ubiquitin ligase and undergoes partial degradation in the proteasome (Jiang and Struhl, 1998). The product of this partial degradation (CiR), comprising the N-terminal repressor domain and the DNA binding domain, represses transcription of Hh target genes (Aza-Blanc et al., 1997). Low levels of Hh inhibit the production of CiR, but are not sufficient to turn the full-length Ci into a functional activator (CiA). Only a high level of Hh can activate the Ci activator function. Therefore, a null mutation in *Drosophila ci* results in de-repression of some Hh target genes normally repressed by CiR, as well as inactivation of other Hh target genes whose transcription depends on CiA (Methot and Basler, 2001).

Consistent with the important roles of phosphorylation in both Smo and Ci activity regulation, the dephosphorylation of these proteins are also important for Hh pathway regulation. Protein phosphatase 4 (PP4) specifically dephosphorylates Smo and plays a negative role in Hh signaling, whereas protein phosphatase 2a (PP2a) appears to play an activating role in the pathway by desphosphorylating Ci (Nybakken et al., 2005; Jia et al., 2009a).

In the absence of Hh, Ci is associated with the kinesin-like protein Costal2 (Cos2) and a serine/threonine kinase Fused (Fu) (Jia et al., 2003; Lum et al., 2003b; Ogden et al., 2003; Ruel et al., 2003). Cos2 plays both positive and negative roles in Hh signaling. In the absence of Hh, Cos2 tethers Ci to the microtubule cytoskeleton and promotes the production of CiR by recruiting PKA, CKI and GSK3 (Zhang et al., 2005). In the presence of Hh, Cos2 physically interacts with the C-terminal tail of Smo, allowing maximal activation of Ci by Smo.

Suppressor of Fused (Sufu) is also associated with Ci (Jia et al., 2003; Lum et al., 2003b; Ogden et al., 2003; Ruel et al., 2003) (Fig. 2). Sufu is not essential for Hh signaling in *Drosophila* but loss of *Sufu* can restore the Hh signaling in *fu* mutants, suggesting that Fu promotes Hh signaling by inhibiting Sufu function (Preat, 1992). Molecularly, Sufu appears to sequester Ci in the cytoplasm in the absence of Hh (Methot and Basler, 2000; Wang et al., 2000b).

Smo activation leads to the phosphorylation of Cos2 and Fu, and the dissociation of Ci from this multiprotein complex (Jia et al., 2003; Lum et al., 2003b; Ogden et al., 2003; Ruel et al., 2003). However, overexpressed Sufu appears to remain associated and translocated together with Ci into the nucleus in the presence of Hh (Sisson et al., 2006). Hh signaling promotes the phosphorylation of Sufu, which may hinder its ability to inhibit Ci activator activity.

Although direct physical association between Smo and the Ci-containing complex through Cos2 has been widely

recognized, how Smo activation leads to the activation of Ci is still elusive. Consistent with Smo being a G protein-coupled receptor, it was found that an activated form of $G\alpha_i$ activates Hh signaling downstream of Smo (Ogden et al., 2008). However, increasing the level of cAMP leads to a severe loss of Hh signaling phenotype only when Smo activity is reduced simultaneously, suggesting that $G\alpha_i$-mediated downregulation of cAMP cannot account for all Hh signaling activities.

Signal transduction from Smo to Gli proteins in vertebrates

In mammals, the bipartite function of *Drosophila* Ci is divided among three Gli family proteins, Gli1, Gli2 and Gli3 (Matise and Joyner, 1999) (Fig. 3). Gli1 is an obligate transcriptional activator because it lacks much of the N-terminal repressor domain and does not appear to be subject to proteolytic processing. Both Gli2 and Gli3 undergo proteolytic processing in vivo, but Gli3 is much more efficiently processed than Gli2 (Wang et al., 2000a; Pan et al., 2006). Consequently, Gli2 appears to act primarily as a transcriptional activator during vertebrate development, whereas Gli3 appears to be a major repressor.

Despite showing the strongest activator activities in gain-of-function analyses, loss of Gli1 function in vivo does not disrupt Hh pathway activation and normal development (Park et al., 2000; Bai et al., 2002). This is consistent with the fact that the transcription of *Gli1* is dependent on Hh pathway activation, making it an unlikely effector of the pathway at the onset of the signaling (Bai et al., 2004). Loss of *Gli2* results in multiple defects, including abnormal ventral patterning of the spinal cord and somites, consistent with its role as the primary positive mediator of Hh signaling (Ding et al., 1998; Matise et al., 1998). *Gli3* mutants exhibit severe polydactyly in all limbs, supporting its role as the major repressor (Hui and Joyner, 1993). Interestingly, mouse embryos doubly mutated for *Gli2* and *Gli3* (equivalent to *Gli1/2/3* triple mutants as *Gli1* is not expressed in these mutants) exhibit more severe defects in the ventral spinal cord than *Gli2* single mutants, suggesting that the activator function of both Gli2 and Gli3 contributes to the normal development of the CNS (Bai et al., 2004; Lei et al., 2004).

The primary cilium plays a unique role in the regulation of Gli activities in mammals. As discussed above, Smo activation in mammals is dependent on cilia, but loss of cilia does not lead to the same defects as seen in *Smo* mutants (Zhang et al., 2001; Huangfu et al., 2003; Liu et al., 2005). Mouse *Smo* mutants have a severe loss of almost all ventral spinal cord cell types, whereas a complete loss of cilia only leads to the loss of floor plate and V3 interneurons, two cell types that require Gli activator activities. The motor neurons, V2 and V1 interneurons are all present in the cilia mutant spinal cords, but are no longer segregated into distinct groups. This relatively mild phenotype is not due to an incomplete loss of Smo activation because loss of Shh or Smo in cilia mouse mutants does not exacerbate the spinal cord defects of cilia mutants (Huangfu and Anderson, 2005; Liu et al., 2005). Strikingly, cilia mutants that survive to later stages exhibit polydactyly in the limb buds, which is opposite to the great loss of digits in *Shh* mutants (Chiang et al., 2001; Kraus et al., 2001; Liu et al., 2005).

The unique Hh signaling defects seen in cilia mutants appear to suggest that not only the Gli activator, but also the Gli repressor activities are compromised in the absence of cilia. Indeed, cilia mutants closely resemble *Gli2;Gli3*, *Shh;Gli3* and *Smo;Gli3* double mutants in which both activator and repressor activities of Gli are compromised or abolished (Litingtung and Chiang, 2000; Litingtung et al., 2002; Wijgerde et al., 2002; Bai et al., 2004; Lei et al., 2004). Moreover, Western blot analysis indicates that the loss of cilia leads to reduced proteolytic processing of Gli3, providing a molecular basis for the genetic defects implicating compromised Gli repressor activities (Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005; May et al., 2005).

Consistent with the roles of cilia in Gli activation and proteolytic processing, all three mammalian Gli proteins are localized to the tips of cilia (Haycraft et al., 2005; Chen et al., 2009; Zeng et al., 2010b) (Fig. 3). Interestingly, full-length, but not the truncated Gli proteins mimicking the processed form, show ciliary accumulation, suggesting that Gli processing may occur after full-length Gli proteins are translocated to the cilia (Haycraft et al., 2005; Zeng et al., 2010b).

Similar to Ci, the proteolytic processing of Gli2 and Gli3 also depends on their phosphorylation by PKA, CKI and GSK3, as well as βTRCP, the mammalian homolog of Slimb (Wang et al., 2000a; Pan et al., 2006). A recent study indicated that PKA is enriched at the base of the cilia, consistent with the roles for cilia in Gli processing (Barzi et al., 2010). Interestingly, PKA activation diminishes ciliary localization of Gli2 and Gli3 but only moderately reduces that of Gli1 (Wen et al., 2010; Zeng et al., 2010b). Replacing Serines in the PKA sites with Alanines such that Gli2 can no longer be phosphorylated does not abolish the effects of PKA activation on Gli2 ciliary trafficking. Furthermore, mimicking the phosphorylated state by replacing those Serines with Aspartic acids does not prevent Gli2 from entering cilia. Therefore, PKA may regulate Gli2 ciliary localization indirectly through phosphorylation of an unknown factor.

The loss of Gli activator activities in cilia mutants coincides with elevated levels of full-length Gli2 and Gli3 proteins, suggesting an inhibitory mechanism that inactivates Gli proteins (Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005; May et al., 2005). A strong candidate for such a mechanism is mammalian Sufu. In striking contrast to its *Drosophila* counterpart, whose inhibitory roles in Hh signaling can only be revealed on *Fu* mutant background, the mammalian Sufu is absolutely required for the negative regulation of Hh signaling and normal embryonic development (Preat, 1992; Cooper et al., 2005; Svard et al., 2006).

Mammalian Sufu directly interacts with all three Gli family members and are believed to inhibit Gli activator activities by preventing their nuclear import (Ding et al., 1999; Pearse et al., 1999; Stone et al., 1999). It was reported that Sufu also has a nuclear role in direct repression of Gli mediated transcription (Cheng and Bishop, 2002; Paces-Fessy et al., 2004). However, this nuclear role of Sufu has been challenged in recent studies (Chen et al., 2009; Humke et al., 2010).

Although genetic studies clearly indicated that Sufu is a negative regulator of Hh signaling, biochemical studies suggest that Sufu also protects Gli2 and Gli3 from proteasome-based degradation (Chen et al., 2009; Zhang et al., 2009) (Fig. 3). This appears to be achieved through its competition with Spop/Cul3-containing ubiquitin ligase for binding with Gli proteins. It has been proposed that by protecting Gli2 and Gli3 proteins, Sufu may play an unappreciated positive role in Hh signaling in mammals. However, such a role has yet to be addressed *in vivo*.

Mammalian Sufu is localized to the tips of primary cilia, similar to the Gli family proteins (Haycraft et al., 2005; Chen et al., 2009; Tukachinsky et al., 2010; Zeng et al., 2010b) (Fig. 3). We and others found that the physical interaction between Sufu and Gli proteins remains intact when cilia are absent, raising the possibility that Sufu keeps full-length Gli proteins from being activated (Chen et al., 2009; Jia et al., 2009b). Indeed, when we knock down Sufu in cilia mutant cells, a Gli-responsive reporter is activated. More convincingly, in *Sufu;Ift88* and *Sufu;Kif3a* double mutant embryos, in which Sufu is removed in the absence of cilia, Hh pathway is ubiquitously activated, providing strong support for a role of Sufu in silencing Gli proteins in the absence of cilia.

Interestingly, translocation of Sufu into cilia is dependent on Gli proteins (Tukachinsky et al., 2010; Zeng et al., 2010b). This suggests that Sufu and Gli proteins form a complex prior to their ciliary entry and is consistent with the observed interaction between Sufu and Gli proteins in the absence of cilia.

Drosophila Cos2 is a unique kinesin-like protein that appears to lack a functional motor domain (Robbins et al., 1997; Sisson et al., 1997). Among vertebrate kinesin-like proteins, Kif7 and Kif27 share the highest homology with Cos2, but they both appear to be functional kinesin subunits. Zebrafish Kif7 is an important negative regulator of Hh signaling (Tay et al., 2005). In mammals, an *in vitro* study suggested that Kif7 is not essential for Hh signaling (Varjosalo et al., 2006). However, mutant mouse analyses revealed an essential negative role of Kif7 in Hh pathway in vivo (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009). Kif7 directly interacts with both Smo and Gli proteins, consistent with a conserved role of mediating local interaction between Smo and Gli proteins (Cheung et al., 2009; Endoh-Yamagami et al., 2009). Kif7 is enriched at the tip and base of the primary cilium, and it appears to play a positive role in Gli3 ciliary localization (Endoh-Yamagami et al., 2009; Liem et al., 2009).

Fused is an essential component of the cytoplasmic Hh signaling complex in *Drosophila*. In contrast, mouse Fused does not appear to regulate Hh signaling (Wilson et al., 2009b). *Fu* mutant mice were born with no defects suggestive of Hh signaling, but die by postnatal day (P) 21 with hydrocephalus and respiratory tract inflammation (Chen et al., 2005; Merchant et al., 2005). Interestingly, mammalian Fu protein plays a specific role in regulating the formation of the central pair of microtubules in motile cilia axoneme, which is essential for the motility of these cilia (Wilson et al., 2009b). Kif27, the other mouse homolog of Cos2, appears to be involved in the same process and physically interacts with Fu.

Finally, additional mechanisms of Gli activity regulation have been revealed recently. According to a recent report, Gli1 and Gli2 are acetylated proteins and Hh activates their transcriptional activities by promoting their deacetylation (Canettieri et al., 2010). Pias1-mediated Small ubiquitin-related modifier (SUMO)-1 conjugation appears to be another important modification of Gli proteins necessary for their full activation (Cox et al., 2010). PKA activation reduces Gli SUMOylation. PKA may also represses Gli activities by promoting interactions between Gli proteins and 14-3-3 (Asaoka et al., 2010). It is important to substantiate these biochemical findings with mutational analyses and to uncover the subcellular locations of these events.

Transcription-independent effects of Hh signaling

In addition to its critical roles in cell fate determination, neural progenitor proliferation, and neuronal differentiation during spinal cord development, Shh also plays an essential role in regulating axon projection. In *in vitro* assays, Shh protein can induce axon-turning response in both rat spinal cord explants and isolated frog commissural neurons (Charron et al., 2003). This response requires Smo activation because cyclopamine-treated neurons fail to turn their axons in response to Shh, and lineage-specific inactivation of Smo in commissural neurons leads to defects in the projection of commissural axons. Boc is also involved in Shh-guided axon projection (Okada et al., 2006). Interestingly, the axons turn within one hour after being exposed to Shh, suggesting that this response does not involve Gli-mediated transcriptional response. Indeed, a recent study suggested that an alternative, Src-family kinase mediated pathway underlies this unique function of Shh (Yam et al., 2009).

Perspective

Three decades have passed since the initial description of *Hedgehog*, the *Drosophila* segment polarity mutant that opened up the gate to all the important discoveries about this critically important signaling pathway. Thirty years of intensive studies on the molecular mechanisms underlying

Hh signaling have resulted in successful clinical trials on cancer treatment with Smo antagonists (Von Hoff et al., 2009; Yauch et al., 2009). However, our understanding of the pathway regulation is far from complete and far from meeting the need for effective disease treatment. One recent report on the quick development of medulloblastoma with resistance against the Smo antagonist GDC-0449 in a clinical trial highlighted the need to develop drugs against multiple targets in the pathway (Yauch et al., 2009). Here we list some of the many challenges that in our opinion deserve attention in future studies.

On the production, distribution and reception of Hh ligands

The roles of dual-lipid modification of Hh in the distribution and activity of the protein are still not well understood. Apparently, these modifications do more than simply increasing hydrophobicity and facilitate multimer formation because removing cholesterol and palmitate moiety affects Hh signaling differently (see the section "Production and distribution of Hh"). It is also striking that the multimeric Shh exhibits 30 times more potency than monomers in *in vitro* reporter assays, but we have yet to discover the mechanism underlying this difference (Zeng et al., 2001; Chen et al., 2004). Why do heparan sulfate proteoglycans play opposite roles in the distribution of Hh family proteins in *Drosophila* and vertebrates? Finally, why do Disp and Ptc, two proteins with high sequence and structural homology, serve completely different functions in Hh signaling? These are all questions we have to address to better understand how Hh proteins are released, distributed in tissues and received by target cells.

On the nature of Smo activation

It is clear now that Smo activation in both *Drosophila* and vertebrates involves changes in its conformation and subcellular localization (see the section "Hh-dependent Smo activation"). However, how these changes are elicited is not clear. Several mechanisms, including the roles of specific lipid composition of the membrane, have been proposed in *Drosophila* and need to be further investigated. Also, whether similar mechanisms exist in vertebrate is not known. Finally, the roles of G-protein in Smo-mediated pathway activation, especially in vertebrates, are still controversial and need further investigation.

On the nature of Ci/Gli activation

One long-standing question in the understanding of Hh signaling is what constitutes the activated state of Ci/Gli proteins. Some recent studies appeared to suggest that the dissociation from Sufu allows the activation of Gli in response to Hh (see the section "Signal transduction from Smo to Gli proteins"). However, replacing Gli3 with a repressor form almost completely restores the D/V patterning of the mouse spinal cord in the absence of Sufu, suggesting that cells in the ventral spinal cord may still respond to positional cues in the absence of Sufu (Wang et al., 2010). If Shh were part of this positional cue, it would suggest that Hh signaling could bypass Sufu to elicit at least some responses in target cells.

Also, it is not clear whether *Drosophila* Sufu dissociates with Ci upon Hh pathway activation. Overexpression studies suggested that they are translocated to the nucleus together in the presence of Hh (Sisson et al., 2006). It is important to address whether endogenous Sufu and Ci exhibit the same behavior.

On the roles of the primary cilia in Hh signaling

Among all the components of the Hh signal transduction pathway, the roles of the primary cilium are probably the least understood. It is now widely accepted that many intracellular Hh signaling events may occur inside the cilia or at the base of the cilia. However, the simple view of cilia being a gathering place for the Hh signaling components may not explain everything. For example, simply keeping Smo inside cilia by treating cells with cyclopamine or disrupting retrograde IFT does not lead to the activation of Smo (Huangfu and Anderson, 2005; May et al., 2005). Some proposed a 2-step activation mechanism, and others proposed that the dynamic trafficking of Smo is critical for its activation. However, it is possible that the removal of a negative regulator of Smo from the cilia is critical for Smo activation. These possibilities all need to be further addressed.

It is also interesting that mutations in mouse *Thm1* and *Ift122* that disrupt retrograde IFT result in constitutive activation of Hh pathway (Tran et al., 2008; Qin et al., 2011). Tulp3, a negative regulator of Hh pathway in mouse, appears to interact with components of IFT-A complex that are involved in retrograde transport (Cameron et al., 2009; Norman et al., 2009; Patterson et al., 2009; Mukhopadhyay et al., 2010). In contrast, removing the retrograde IFT motor Dynein appears to compromise Hh signaling (Huangfu and Anderson, 2005; May et al., 2005). These latest findings further suggested the complexity of cilia-mediated Hh signaling in mammals and pointed to the need for more detailed studies on how the trafficking and activities of various Hh pathway components are regulated inside the cilia.

Finally, Shh appears to regulate the ciliary accumulation of endogenous Ptc, Smo, Sufu and Gli proteins, but the molecular mechanisms underlying this regulation remain a mystery. PKA regulates the ciliary accumulation of Smo, Sufu and Gli proteins; however, it enhances Smo ciliary localization but inhibits Gli and Sufu ciliary localization. Therefore, it is unlikely that Shh regulates the ciliary localization of all these proteins through global regulation

of PKA activity. In addition, our mutational analyses have yet to establish a correlation between Gli2/3 phosphorylation and their ciliary localization, suggesting that more work is needed to reveal the molecular mechanisms by which PKA regulates the ciliary localization of Gli proteins.

Although the current model (Fig. 3) suggests that Hh pathway activation involves the trafficking of multiple Hh pathway components into or out of cilia, there is no direct evidence showing that proper localization of these proteins (except for Smo) is required for Hh pathway activation. The greatest caveat in addressing this question is the difficulty in altering ciliary localization of these proteins with the confidence that other regulatory mechanisms are not affected. Therefore, a better understanding of the mechanisms underlying the ciliary localization of these proteins is essential for further functional analyses.

Finally, what is the evolutionary history of cilia-related Hh signaling? One possibility is that cilia are co-opted as part of the Hh signaling machinery along with the emergence of vertebrates. Alternatively, the connection between Hh signaling and cilia may have been lost during the evolution of modern day insects. A recent study in planarian, a species divergent from both insects and vertebrates, showed that Fu and Cos2 homologs are both involved in ciliogenesis, but not in Hh signaling (Rink et al., 2009). This appears to suggest that the association between Hh signaling and cilia can be dated back to the common ancestors of worms, insects and vertebrates. Even more intriguing, what is the advantage of keeping the connection between cilia and Hh in vertebrates, and why can insects fare so well after losing such a connection?

Acknowledgements

We thank Westley Heydeck, Huiqing Zeng and Jinling Liu for critically reading the manuscript. We apologize to colleagues whose work we failed to include in this short review because of the huge amount of information published in this field. Research activities in our laboratory are supported by grants from NSF, American Heart Association, PKD foundation and a new laboratory start-up fund from the Pennsylvania State University.

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