

Annual Review of Cell and Developmental Biology
Sending and Receiving
Hedgehog Signals

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Abstract

Communication between cells pervades the development and physiology of metazoans. In animals, this process is carried out by a relatively small number of signaling pathways, each consisting of a chain of biochemical events through which extracellular stimuli control the behavior of target cells. One such signaling system is the Hedgehog pathway, which is crucial in embryogenesis and is implicated in many birth defects and cancers. Although Hedgehog pathway components were identified by genetic analysis more than a decade ago, our understanding of the molecular mechanisms of signaling is far from complete. In this review, we focus on the biochemistry and cell biology of the Hedgehog pathway. We examine the unique biosynthesis of the Hedgehog ligand, its specialized release from cells into extracellular space, and the poorly understood mechanisms involved in ligand reception and pathway activation at the surface of target cells. We highlight several critical questions that remain open.



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INTRODUCTION

The Hedgehog signaling pathway is a key communication system between metazoan cells. It is involved in all stages of an animal's life, from embryonic development to maintenance and regeneration of adult tissues. Thus, understanding Hedgehog signaling is crucial to understanding animal development and physiology and, beyond that, to harnessing its power in disease treatment and regenerative medicine.

In this review, we focus on the molecular mechanisms used by cells to send and receive Hedgehog signals (**Figure 1**). We follow the Hh ligand (Hh), from its unique biosynthesis, through its secretion from producing cells, to its reception by target cells. The bulk of the evidence that we cite comes from three model organisms in which the Hedgehog pathway is best understood: the fruit fly *Drosophila melanogaster*, the house mouse *Mus musculus*, and the zebrafish *Danio rerio*. Although Hedgehog signaling operates in all these organisms, evolutionary divergence has resulted in the expansion of certain protein families. For example, whereas fruit flies have one gene coding for one Hh, mice have three and zebrafish have six (see sidebar titled Hedgehog Ligand Homologs).

To avoid confusion, in cases in which the functional mechanism is conserved between homologs, we refer to the whole set by a single name and a single discussion. So, by Hh we designate all Hh homologs and discuss them as a unit. In cases in which the functional mechanism is known to, or is suspected to, diverge between homologs, we draw special distinction. This often occurs in the discussion of vertebrate and invertebrate homologs.

In addition to differences between homologs in different species, there appear to be some differences in the pathway's behavior in different tissues of the same organism. We note such differences when appropriate, but, as our goal is to outline the general principles of the pathway, the reader is advised to consult species- or tissue-specific literature for further details.

HEDGEHOG LIGAND HOMOLOGS

Whereas the genome of the fruit fly has a single gene coding for Hh, *hedgehog* (*hh*), higher organisms have several. For example, the mouse has three orthologous Hh genes: *Sonic hedgehog* (*Shh*), *Desert hedgehog* (*Dhh*), and *Indian hedgehog* (*Ihh*). In zebrafish, due to a genome duplication event, there are as many as six paralogs: *sonic hedgehog a* (*shba*), *sonic hedgehog b* or *tiggywinkle hedgehog* (*shbb/twhh*), *indian hedgehog a* (*ihba*), *indian hedgehog b* or *echidna hedgehog* (*ihhb/ebb*), *qiqihar hedgehog* (*qhb*), and *desert hedgehog* (*dhh*). Sequence conservation and biochemical studies show that all these paralogous ligands are processed similarly, being modified with both palmitoyl and cholesteryl moieties. Thus, the differences between paralogs appear to be in the timing and location of expression. A number of excellent reviews summarize tissue-specific expression patterns and functions of these proteins (Ingham et al. 2011, Jeong & McMahon 2002, Varjosalo & Taipale 2008).

OUTLINE OF THE HEDGEHOG SIGNALING PATHWAY

Like other signaling pathways, the Hedgehog pathway consists of a chain of molecular events through which a signal produced by a cell (referred to as the sending cell) controls the behavior of another cell (termed the receiving cell). The sending cell synthesizes Hh and enables its extracellular release in a diffusible form. Once Hh leaves the sending cell, it travels through the extracellular milieu, reaching the surface of the receiving cell. There, Hh interacts with its coreceptor(s) and receptor Patched (Ptc), triggering an intracellular signal transduction cascade, which ultimately results in specific changes in gene expression. The mechanisms underlying Hh synthesis, release from cells, transport, and reception are remarkably complex and subject to numerous regulatory interactions.

Hh is modified with two lipids at its termini: an N-terminal palmitoyl moiety and a C-terminal cholesteryl moiety. The lipid modifications, particularly the cholesteryl moiety, render Hh highly hydrophobic, resulting in strong retention on the plasma membrane of sending cells. This feature of Hh would make it a poor intercellular signaling molecule were it not for a set of factors dedicated to its release. In both flies and vertebrates, the transmembrane protein Dispatched (Disp) is required for Hh release, likely by facilitating its extraction from the plasma membrane. Following interaction with Disp, Hh moves into the extracellular space, complexed with a chaperone. The chaperone ensures Hh solubility by shielding the hydrophobic cholesteryl and palmitoyl moieties from the aqueous environment. With its lipid appendages safely sequestered, Hh and its associated chaperone are then free to diffuse away from sending cells. A number of factors have been described as Hh chaperones, from the Scube family of proteins, to lipoprotein particles, to Hh itself.

Movement of Hh between cells is regulated by numerous factors, including extracellular matrix components [such as negatively charged heparan sulfate proteoglycans (HSPGs)] and cell surface proteins (Cdon/Ihog, Boc/Boi, Gas1, Hhip). In addition to regulating Hh spreading, Cdon/Ihog, Boc/Boi, and Gas1 also facilitate Hh reception as coreceptors. The cell surface protein Ptc ultimately acts as a receptor for Hh.

In contrast to most other ligand–receptor pairs, Hh represses rather than activates Ptc upon binding. Peculiarly, this repression of Ptc results in pathway activation. This is because unliganded Ptc inhibits the G protein–coupled receptor (GPCR)–like protein Smoothed (Smo) (Ingham & McMahon 2001), an essential activator of the cytoplasmic steps of Hedgehog signaling. When bound to Hh, Ptc no longer represses Smo, which leads to Smo activation. How Ptc represses Smo and how Hh represses Ptc are major open questions in the field. Once activated, Smo sets in

Lipoprotein particles:

protein–lipid complexes that transport lipids systemically; composed of a lipid core surrounded by a phospholipid monolayer and apolipoproteins

Heparan sulfate:

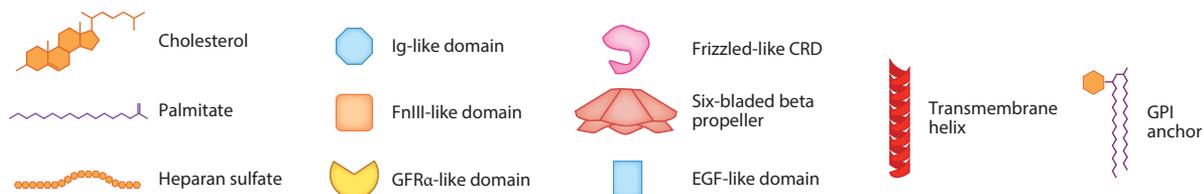
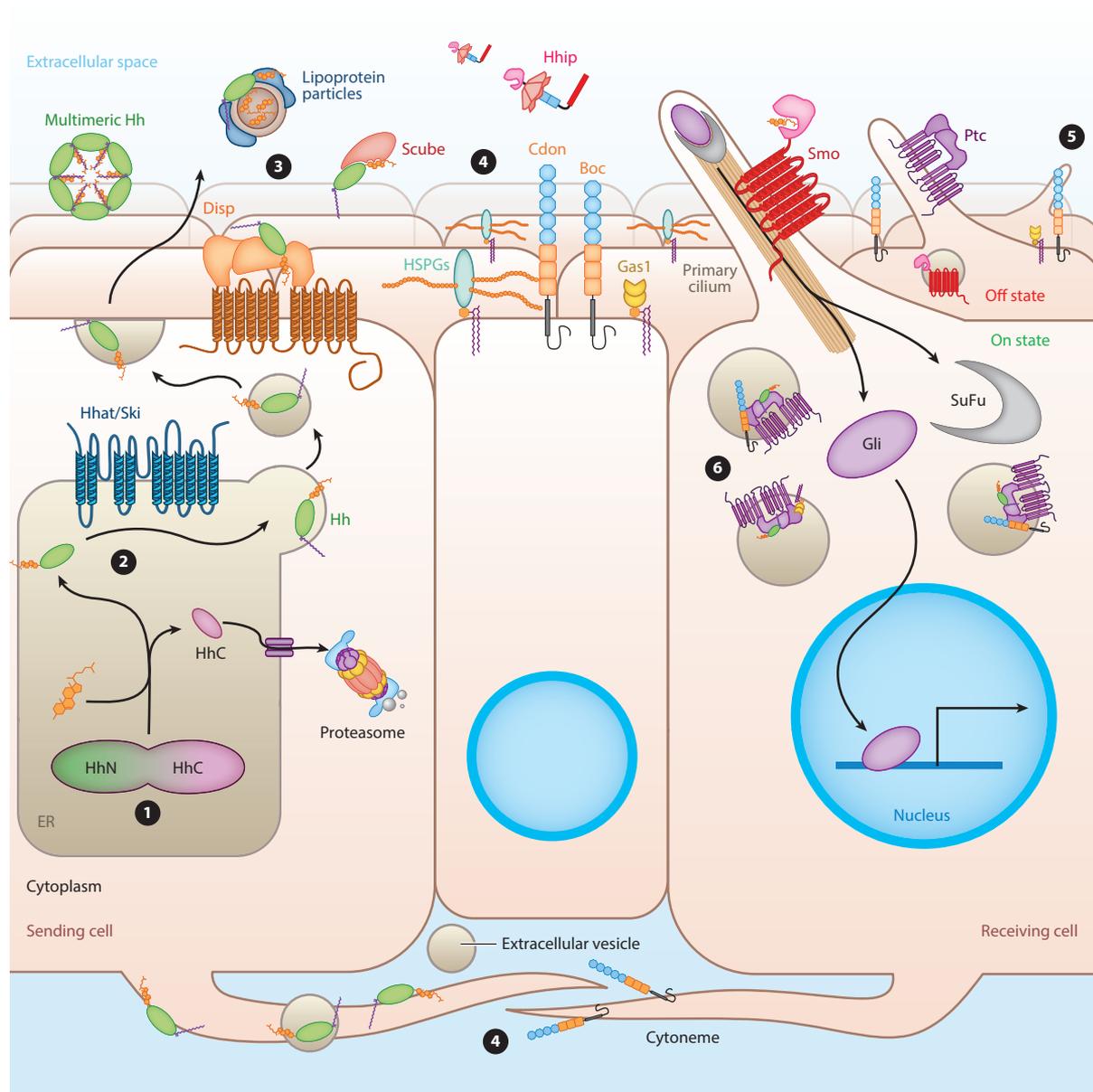
a negatively charged linear polysaccharide

Heparan sulfate proteoglycan (HSPG):

a class of glycoproteins consisting of a protein core attached to heparan sulfate chains

G protein–coupled receptor (GPCR):

a class of seven-pass transmembrane proteins that activate heterotrimeric G proteins in response to ligand binding



motion the downstream steps in signal transduction, causing activation of the Zn-finger family of transcription factors that mediate all transcriptional effects of the Hh pathway, the Gli1–3 proteins in vertebrates, and the Ci protein in flies. The mechanisms involved in Gli/Ci regulation have been reviewed elsewhere (Huangfu & Anderson 2006). Below, we discuss the mechanisms of Hh synthesis, release, transport, and reception in more detail.

Hint/Hog: a protein domain homologous to self-splicing inteins and to the C-terminal domain of Hh proteins

BIOSYNTHESIS OF THE HEDGEHOG LIGAND

Hh is the only protein known to be covalently modified with palmitoyl and cholesteryl moieties. These posttranslational modifications are essential for the function of Hh and can occur independently (Buglino & Resh 2008, Chen et al. 2004), although whether *in vivo* they affect each other's efficiency is unclear (Creanga et al. 2012).

Hh is first synthesized as a two-domain precursor, comprising an N-terminal signaling domain, HhN, and a C-terminal Hint/Hog intein domain, HhC. The HhN domain is preceded by a signal sequence, which targets the precursor for cotranslational translocation into the lumen of the endoplasmic reticulum (ER). Removal of the signal sequence by a signal sequence protease leaves a conserved cysteine as the first amino acid in HhN, a residue critical for the palmitoylation reaction (Pepinsky et al. 1998). Palmitoylation is catalyzed by an ER-resident, membrane-bound, *O*-acyltransferase termed Skinny hedgehog (Ski) in flies (Amanai & Jiang 2001, Chamoun et al. 2001, Chen et al. 2004, Lee & Treisman 2001, Micchelli et al. 2002) and Hh acyltransferase (Hhat) in vertebrates (Buglino & Resh 2008, Konitsiotis et al. 2014). Hhat/Ski catalyzes a thioester exchange reaction between the thiol group of the N-terminal cysteine and palmitoyl-CoA. The cysteine thioester thus formed is thought to rearrange spontaneously via an S-to-N shift, whereby the N α -amine of the cysteine attacks the neighboring thioester, resulting in a stable amide linkage between the palmitoyl moiety and HhN. Specificity of Hhat for HhN is encoded by a short, conserved sequence in HhN that follows the acceptor cysteine; as few as the first six amino acids of HhN are sufficient for Hhat-catalyzed palmitoylation (Buglino & Resh 2008).

Cholesterol modification is unique to Hh and is accomplished autocatalytically by the intein-like activity of the HhC domain of the Hh precursor (Porter et al. 1996a,b). In a first step, the

Figure 1

Key biochemical and cellular events in the Hedgehog signaling pathway. (1) In sending cells, the Hedgehog ligand (Hh) is synthesized as a precursor, with an N-terminal signaling domain (HhN, *green*) and a C-terminal domain (HhC, *purple*). (2) Hh undergoes maturation in the endoplasmic reticulum (ER). Through the self-splicing activity of HhC, HhN is cleaved from the precursor and is covalently modified with cholesterol on its C terminus. HhC is then cleared by ER-associated degradation. HhN is also palmitoylated on its N terminus by the Hhat/Ski acyltransferase. (3) The dually lipidated mature ligand (Hh) is released from sending cell membranes by the synergistic action of Dispatched (Disp, *orange*) and an extracellular chaperone. Potential chaperones include Hh itself, lipoprotein particles, and the Scube family of proteins (*top left*) as well as extracellular vesicles (*bottom left*). (4) Hh moves through the space between sending and receiving cells (*center*) either as a diffusible signal (*top*) or associated with cellular extensions known as cytonemes (*bottom*). Extracellular Hh movement requires heparan sulfate proteoglycans (HSPGs, *teal*) and is controlled by the Hh coreceptors Cdon, Boc, and Gas1, as well as by the secreted protein Hhip. (5) In the absence of ligand, the Hedgehog pathway is inactive (*top right*, background cell): The Hh receptor Patched (Ptc, *purple*) is concentrated in the primary cilium, whereas the downstream transducer Smoothed (Smo, *red*) is sequestered in intracellular compartments (*top right*, background cell). (6) The pathway is activated upon Hh binding to Ptc, a process that requires the assistance of the Hh coreceptors. Hh binding directly inhibits Ptc, relieving its repression of Smo. Ptc bound to Hh then exits the cilium and is degraded while Smo accumulates in the cilium in the active form. Smo then causes activation of Gli transcription factors (*purple*) by relieving their inhibition by the cytoplasmic sequestration factor SuFu (*gray*). Active Gli proteins subsequently move to the nucleus and drive transcriptional activation of Hedgehog pathway target genes. Other abbreviations: CRD, cysteine-rich domain; EGF, epidermal growth factor; FnIII, fibronectin type III; GPI, glycosylphosphatidylinositol; Ig, immunoglobulin.

Protein disulfide isomerase (PDI): an ER-resident enzyme that remodels disulfide bridges to help protein folding in the secretory pathway

thiol group of a catalytic cysteine located at the junction between HhN and HhC reacts with the preceding amide bond, forming an internal protein thioester, which serves as a C-terminally activated form of the HhN domain. In a second step, the 3β -OH group of a cholesterol molecule reacts with the thioester, cleaving the Hh precursor into cholesterylated HhN (in which cholesterol is attached to the C terminus of HhN via an ester linkage) and free HhC. The rate of the intein reaction is independent of Hh precursor concentration, suggesting that the reaction happens *in cis* (Porter et al. 1995). The structure-activity relationship for the modifying sterol shows significant flexibility, and many sterols besides cholesterol can drive Hh modification, as long as they possess a 3β -OH group (Cooper et al. 1998, Mann & Beachy 2000). Binding of the HhC intein to cholesterol is suspected to occur but has not been demonstrated. The presumed sterol-binding site is likely located within the last 60 residues of HhC, as HhC lacking this portion can undergo the first step (internal thioester formation), but not the second step (cholesterol attachment), of the modification reaction (Hall et al. 1997).

Interestingly, autocatalytic cleavage of the Hh precursor by HhC requires the activity of protein disulfide isomerases (PDIs) (Chen et al. 2011). PDIs are ER-resident enzymes that remodel disulfide bridges between cysteine residues to help protein folding in the secretory pathway (Wilkinson & Gilbert 2004). In the case of HhC, the catalytic cysteine first forms a disulfide bridge with another conserved cysteine in HhC; this bond may be required for proper folding of the HhC domain. PDIs then reduce the disulfide bond, making the thiol group of the catalytic cysteine available for the first step of the autoprocessing reaction.

HhC does not appear to have signaling activity, and its only function appears to be in HhN cholesterylation. In fact, after the autoprocessing reaction is completed, free HhC is cleared by ER-associated degradation (ERAD) (Chen et al. 2011). It is unclear how HhC is recognized as a substrate for ERAD, given that ERAD generally recognizes misfolded proteins. Disulfide bridge reduction, which precedes the autocatalytic reaction, may render HhC structurally unstable such that it is recognized by the ERAD machinery and degraded. Interestingly, the HhC domain can target the entire Hh precursor for ERAD. Thus, Hh precursor processing competes with Hh degradation in the ER, which has implications for the etiology of developmental diseases associated with mutations in the human *SHH* gene (Chen et al. 2011). These mutations reduce Hh signaling by impairing Hh cholesterylation, thus favoring Hh precursor degradation over Hh secretion. It remains to be determined whether competition between cholesterylation and ERAD plays a role during normal Hh signaling, perhaps as a mechanism by which cholesterol levels in the ER control the amount of generated ligand.

Dual-lipid modification makes Hh highly hydrophobic and therefore tightly associated with cell membranes. This property seems at odds with the ligand's well-established role in long-range signaling, raising two key questions: (a) How is Hh released from sending cells, and (b) how does Hh move between cells? In the next two sections, we describe how these processes are executed on a molecular level.

HEDGEHOG LIGAND RELEASE FROM CELLS

The Role of Dispatched

The transmembrane protein Disp is required for Hh-dependent tissue patterning, acting specifically in sending cells (Burke et al. 1999, Caspary et al. 2002, Etheridge et al. 2009, Kawakami et al. 2002, Ma et al. 2002, Nakano et al. 2004). Importantly, Disp is not required for Hh-dependent responses in receiving cells (Amanai & Jiang 2001, Ma et al. 2002, Tian et al. 2005), indicating that it has no role in downstream signal transduction. In the absence of Disp, sending cells express

and process Hh correctly, but Hh accumulates on their surface (Burke et al. 1999, Kawakami et al. 2002, Ma et al. 2002), indicating a defect in ligand release. Interestingly, Disp is not required for signaling between sending cells and immediately adjacent receiving cells, indicating a functional distinction between juxtacrine and long-range Hh signaling processes.

Cholesterol modification of Hh is necessary and sufficient for Disp-dependent release (Burke et al. 1999, Ma et al. 2002, Nakano et al. 2004, Tian et al. 2005). Furthermore, Disp acts specifically on cholesterol anchors, as transmembrane- or glycosylphosphatidylinositol (GPI)-anchored variants of Hh are not released by Disp (Burke et al. 1999). Consistent with this specificity, cross-linking experiments using Hh modified with photoreactive cholesterol show that Disp interacts directly with the cholesteryl moiety (Tukachinsky et al. 2012). How Disp participates in Hh mobilization from membranes is unclear. An attractive model is that, in a first step, Disp acts catalytically to extract the cholesteryl anchor from the lipid bilayer. This mechanism is consistent with the homology between Disp and RND pumps of gram-negative bacteria (Ma et al. 2002, Tseng et al. 1999), which use the energy of the proton gradient across the periplasmic membrane to expel lipophilic small-molecule substrates from cells. Then, in a second step, Disp transfers Hh to an extracellular chaperone, ensuring that the cholesteryl anchor is moved without exposure to an aqueous environment; this is reminiscent of the handoff of cholesterol from Niemann-Pick disease protein C2 (NPC2) to Niemann-Pick disease protein C1 (NPC1) during cholesterol egress from lysosomes (Kwon et al. 2009).

It has been proposed that, in addition to playing a role in Hh release, Disp functions in Hh trafficking and sorting within sending cells, although the mechanism is unclear. Different studies report that Disp localizes Hh to either the apical surface (Gallet et al. 2003) or the basolateral surface (Callejo et al. 2011) of sending cells or that it directs Hh to either location indiscriminately (Burke et al. 1999, Etheridge et al. 2009). Disp was proposed to localize to the basolateral surface (Callejo et al. 2011), mediated by a region in its intracellular tail (Etheridge et al. 2009).

Although Disp may mediate Hh extraction from cell membranes, this activity is insufficient to allow for Hh release and transport between cells. This function is accomplished by extracellular Hh chaperones, which function downstream of Disp and solubilize Hh by shielding its lipid modifications. The first chaperones we discuss are the Scube family of vertebrate proteins. Next, we discuss Hh multimerization as a mechanism by which Hh may act as its own chaperone. We then examine the role of lipoprotein particles and extracellular vesicles as Hh chaperones. Finally, we discuss Hh transport at a distance along cellular projections known as cytonemes.

The Scube Family of Extracellular Chaperones

Genetic studies in zebrafish have identified the Scube family of secreted proteins as being implicated in Hedgehog signaling (Hollway et al. 2006, Johnson et al. 2012, Kawakami et al. 2005, van Eeden et al. 1996, Woods & Talbot 2005). Scube proteins are vertebrate specific, and most animals have three paralogs: Scube1–3. Because of functional redundancy, the paralogs are individually dispensable, but collectively they are strictly necessary for Hedgehog signaling in vivo. Epistasis analysis of Scube2 in zebrafish shows that it acts upstream of Hh reception, although transplantation experiments between wild-type and Scube2-null fish embryos indicate that neither sending nor receiving cells require Scube2 for proper signaling, provided that some Scube2-expressing cells are present in the affected signaling domain. These data suggest that Scube proteins act non-cell-autonomously between sending and receiving cells.

Scube proteins are composed of nine epidermal growth factor (EGF)-like repeats, a spacer, three cysteine-rich domains (CRDs), and a CUB domain. Biochemical experiments revealed that, in the presence of Disp, Scube2 enhances the release of Hh into aqueous solution (Creanga et al.

Glycosylphosphatidylinositol (GPI):

a glycolipid attached posttranslationally to the C terminus of certain proteins that anchors them to the plasma membrane

Resistance-nodulation-division (RND):

a class of multipass transmembrane proteins found in bacteria and eukaryotes; in gram-negative bacteria, RND proteins form trimers that localize to the inner bacterial membrane and use the proton-motive force across it to expel toxic ions or small molecules from cells

Extracellular vesicles: implicated in transport between cells; can be derived from intraluminal vesicles of multivesicular bodies (exosomes) or formed by budding from the plasma membrane (ectosomes)

Cytonemes: thin, actin-based cellular projections that help relay signaling molecules between sending cells and receiving cells

2012, Tukachinsky et al. 2012) and that the CRDs and CUB domain are necessary for this activity. Scube2 interacts directly with the cholesteryl anchor of Hh (Tukachinsky et al. 2012), suggesting a simple mechanism for how Scube proteins solubilize Hh. It remains unclear how Disp and Scube2 cooperate during the handoff of Hh from the former to the latter.

Because Scube proteins are vertebrate specific, an open question is whether there exist functionally analogous proteins in invertebrates. One promising candidate is *Drosophila* Shifted, a secreted, EGF-like domain-containing protein required for long-range signaling by Hh (Glise et al. 2005, Gorfinkiel et al. 2005). Alternatively, the vertebrate and invertebrate pathways for Hh release may have diverged during evolution such that vertebrates use Scube proteins as Hh chaperones, whereas flies use alternative mechanisms (see below).

Soluble Hedgehog Ligand Multimers

A long-standing model for Hh release proposes that Hh overcomes the insolubility conferred by lipidation by acting as its own chaperone, forming multimers that shield the lipid moieties from the aqueous environment. This model is based on the observation of high-molecular weight species of Hh in media conditioned by Hh-expressing cells (Chen et al. 2004, Gallet et al. 2006, Goetz et al. 2006, Palm et al. 2013, Zeng et al. 2001). As the species initially observed was approximately 120 kDa in size, roughly six times the molecular weight of Hh, it was proposed that Hh forms a hexamer. However, the size of Hh species varies between different sources, from ~150 kDa for vertebrate Shh to greater than 600 kDa for *Drosophila* Hh (Chen et al. 2004). The wide range of observed sizes suggests that high-molecular weight Hh species may represent complexes between Hh and other proteins, rather than multimers of Hh. Determining the composition of high-molecular weight Hh species should help answer this question.

A direct role for Disp in the production of Hh multimers has not been established. Some studies propose that the multimeric Hh isolated biochemically may correspond to the large punctate structures in which Hh is observed by microscopy *in vivo*; such studies indirectly suggest a role for Disp in multimer release, as the large punctate structures appear to depend on Disp (Burke et al. 1999; Gallet et al. 2003, 2006; Porter et al. 1996a; Tabata & Kornberg 1994; Taylor et al. 1993). However, the structures may consist not of Hh multimers, but rather of Hh clustered by Hh-binding proteins (Vyas et al. 2008).

Hedgehog Ligand Release by Lipoproteins and Extracellular Vesicles

Hh can be efficiently released from cells by lipoprotein particles (Palm et al. 2013, Panáková et al. 2005), an effect perhaps driven by the interaction between lipids in the particles and lipid moieties of Hh. This interaction is not specific for Hh, as lipoproteins also release other lipidated ligands, such as Wnt proteins (Panáková et al. 2005). It is unclear, however, what role lipoproteins play in Hedgehog signaling, as they do not appear to be required for signaling *in vivo* (Panáková et al. 2005). It also remains to be determined whether and how release of Hh by lipoproteins requires Disp. Unexpectedly, Hh released by lipoproteins has low activity in signaling assays (Palm et al. 2013), and exogenous addition of lipoproteins inhibits Hh signaling. These findings raise the question of how Hh released on lipoproteins overcomes this inhibition.

Recently, several groups have proposed that, at least in flies, Hh is released loaded on extracellular vesicles (Gradilla et al. 2014, Matusek et al. 2014, Parchure et al. 2015). Supporting this model, in wing disks, punctate structures with extracellular Hh colocalize with CD63, a marker of extracellular vesicles (Gradilla et al. 2014). Immuno-electron microscopy of wing discs (Gradilla et al. 2014) and of high-speed pellets from Hh-conditioned media (Matusek et al. 2014) reveals

vesicles containing Hh, Disp, and the Hh coreceptor Ihog. Such vesicles also contain exosomal markers and components of the endosomal sorting complex required for transport (ESCRT). Furthermore, disruption of specific ESCRT proteins affects long-range Hh signaling in the wing disc and causes apical accumulation of Hh in sending cells. Although there is no agreement on the cellular compartment—plasma membrane or multivesicular bodies—from which these extracellular vesicles are derived, different studies propose a potential trafficking function for Disp in the biogenesis of Hh-containing exovesicles (Callejo et al. 2011, D'Angelo et al. 2015). Further study is required, particularly in vertebrate systems, to ascertain a role for Disp in the production of such extracellular vesicles and to confirm whether the isolated Hh-containing vesicles have signaling activity.

Endosomal sorting complex required for transport (ESCRT):

a set of cytoplasmic protein complexes that cooperate to drive membrane budding away from the cytosol, including biogenesis of multivesicular bodies

Multivesicular bodies:

endosomes containing intraluminal vesicles generated by inward budding of their limiting membrane

Glypican: a major class of HSPG that is membrane associated via a GPI anchor

Hedgehog Ligand Transport Along Cytonemes

Cytonemes are long and thin (up to 200 μm in length but only $\sim 0.2 \mu\text{m}$ in diameter) actin-based cellular extensions (Ramírez-Weber & Kornberg 1999). Cytonemes have been proposed to mediate Hh transport to distant cells on the basis of the observations that they project from the basal surface of sending cells in the *Drosophila* wing disc and that they host Hh, Disp, and Ihog (Callejo et al. 2011, Gradilla et al. 2014). Unlipidated HhN and inactive Disp are absent from cytonemes. Furthermore, functional experiments show a correlation between cytoneme length and range of Hh signaling, consistent with the idea that cytonemes mediate Hh transport. A recent study supports the claim that Hh is transported on cytonemes in vertebrates as well (Sanders et al. 2013), suggesting that this mechanism is general.

Although cytonemes may provide a solution to the problem of Hh transport at a distance, it is unclear how Hh moves from the cytonemal membrane to the surface of the receiving cell. It is not known whether Hh chaperones are implicated in this step. In *Drosophila*, Hh-positive exovesicles may associate with cytonemes and move along them (Gradilla et al. 2014), suggesting a role for vesicles in releasing Hh from cytonemes. Another possibility is that Hh transfer from cytonemes to receiving cells is similar to Hh movement between cells during juxtacrine Hh signaling, a process that remains poorly understood mechanistically.

HEDGEHOG LIGAND TRANSPORT

The Role of Heparan Sulfate Proteoglycans

On the way to distant receiving cells, Hh must traverse a dense extracellular environment. This transport process is thought to be facilitated by HSPGs (Bellaiche et al. 1998, Han et al. 2004, Lin et al. 2000, Rubin et al. 2002, The et al. 1999), which are glycoproteins containing one or more heparan sulfate glycosaminoglycan chains (Sarrazin et al. 2011). The role of HSPGs in Hedgehog signaling was discovered by examining mutants lacking specific glycosyltransferases and polymerases, enzymes responsible for heparan sulfate synthesis and transfer. For example, Hedgehog signaling is severely attenuated in fruit fly mutants lacking the glycosyltransferase *tout-velu* (*ttv*) or the UDP-glucose dehydrogenase *sugarless* (Bellaiche et al. 1998, Gallet et al. 2003, Häcker et al. 1997, The et al. 1999). Similar results are observed in tissues lacking exostoses, which are vertebrate homologs of *ttv* (Lin 2004, Lin et al. 2000).

Further evidence for the importance of HSPGs in Hedgehog signaling comes from examining mutants lacking the HSPG protein scaffold. In *Drosophila*, a cell-bound HSPG, the Dally-like glypican, is required for signaling in vivo and in vitro and has non-cell-autonomous as well as cell-autonomous roles (Desbordes & Sanson 2003, Gallet et al. 2008, Lum et al. 2003, Yan et al. 2010).

In vertebrates, the glypicans GPC1, GPC4, and GPC6 promote Hedgehog signaling, whereas GPC2, GPC3, and GPC5 appear to inhibit signaling (Campos-Xavier et al. 2009, Capurro et al. 2008, Williams et al. 2010).

It is thought that HSPGs promote Hedgehog signaling by facilitating Hh transport between cells. This model is supported by two observations. First, Hh accumulates on the surface of cells in the absence of heparan sulfate synthesis (Bellaiche et al. 1998, Gallet et al. 2003, Han et al. 2004). Second, Hh binds heparan sulfate glycosaminoglycans (Chan et al. 2009, Chang et al. 2011, McLellan et al. 2006, Rubin et al. 2002, Whalen et al. 2013) with low-micromolar affinity.

The extent of coordination between Hh release and transport is unclear. Specifically, whether and how Hh chaperones interact with HSPGs and other components of the extracellular milieu are open questions.

Hhip: An Extracellular Hedgehog Ligand Antagonist

Whereas HSPGs facilitate extracellular Hh transport, the vertebrate protein Hhip antagonizes it (Chuang & McMahon 1999, Holtz et al. 2015, Kwong et al. 2014). Initially believed to be a transmembrane (Chuang & McMahon 1999) or GPI-anchored protein (Bosanac et al. 2009), Hhip was recently shown to be secreted and to associate with the extracellular matrix by binding HSPGs (Holtz et al. 2015). Hhip is composed of a basic CRD, a six-bladed beta propeller domain, and two EGF-like repeats. The CRD mediates binding to HSPGs, whereas the beta propeller domain binds vertebrate Shh (Bishop et al. 2009, Bosanac et al. 2009) with low-nanomolar affinity. The Hhip-binding surface of Shh overlaps with the surface thought to interact with the Ptc receptor, explaining why Hhip acts as a negative regulator of Hh signaling via ligand sequestration.

THE FUNCTION OF CORECEPTORS IN HEDGEHOG LIGAND RECEPTION

On the surface of receiving cells, Hh must ultimately bind to its receptor, Ptc. This process is facilitated by a set of membrane proteins known as Hh coreceptors, consisting of the immunoglobulin superfamily (IgSF) members Cdon/Ihog and Boc/Boi (Okada et al. 2006, Tenzen et al. 2006, Yao et al. 2006, Zhang et al. 2006) and the GPI-anchored protein Gas1 (Allen et al. 2007, Lee & Fan 2001, Martinelli & Fan 2007). IgSF coreceptors are found in both invertebrates (Ihog and Boi) and vertebrates (Cdon and Boc), whereas Gas1 is present only in vertebrates.

Coreceptors are collectively necessary for Hedgehog pathway activation (Allen et al. 2011, Camp et al. 2010, Yan et al. 2010, Zheng et al. 2010), but due to redundancy, they are individually dispensable. Further evidence for the role of coreceptors comes from experiments showing that their overexpression induces a cell-autonomous increase in Hh signaling both in vivo (Allen et al. 2011, Martinelli & Fan 2007, Tenzen et al. 2006) and in cell culture (Yao et al. 2006, Zhang et al. 2006). Importantly, cell-autonomous pathway activation depends on Hh, suggesting that the coreceptors facilitate ligand reception rather than promote downstream signaling through other mechanisms.

The IgSF coreceptors are type I transmembrane proteins. Their extracellular domains are composed of a variable number of Ig-like repeats, followed by fibronectin type III (FnIII) repeats. Binding between IgSF coreceptors and ligand is mediated by FnIII repeats. Surprisingly, Hh binds different FnIII repeats in different IgSF orthologs. For example, Hh binds to the first FnIII repeat in fruit fly Ihog (Yao et al. 2006, Zheng et al. 2010) and to the third FnIII repeat of vertebrate Cdon and Boc (Okada et al. 2006, Tenzen et al. 2006, Yao et al. 2006). Orthologous IgSF coreceptors

also differ in cofactor requirement for Hh binding: Ihog requires heparan sulfates (McLellan et al. 2006), whereas Cdon and Boc require calcium (McLellan et al. 2008).

Gas1 is a GPI-anchored membrane protein that consists of two CRDs (Cabrera et al. 2006, Rosti et al. 2015) followed by a flexible linker (Stebel et al. 2000). In contrast to binding between Hh and IgSF coreceptors, binding between Hh and Gas1 has not been thoroughly studied. A Gas1 point mutant defective in Hh binding has been identified (Pineda-Alvarez et al. 2011). Furthermore, mutagenesis experiments suggest that Gas1 likely binds a Hh surface different from the one bound by IgSF coreceptors, Ptc, and Hhip (Martinelli & Fan 2009).

In addition to binding Hh, the coreceptors also bind Ptc in both vertebrates and invertebrates. This interaction depends on the second FnIII repeat in Ihog (Zheng et al. 2010) and on the first and second FnIII repeats in Cdon/Boc (Izzi et al. 2011). Gas1 also binds Ptc, but which part of Gas1 is involved has not been defined. On the Ptc side, coreceptor binding requires the first extracellular loop of Ptc. Cdon/Boc and Gas1 appear to bind to the same site on Ptc, indicating that Ptc is bound either to an IgSF coreceptor or to Gas1 (Izzi et al. 2011). Interestingly, coreceptor binding to Ptc does not depend on Hh, suggesting that the coreceptors and Ptc may form a holocomplex, which may be the functionally relevant form of the Hh receptor in vivo (Zheng et al. 2010). Cdon and Boc, however, have not been observed at the primary cilium (Song et al. 2015) where Ptc localizes in vertebrates, suggesting that at least these two coreceptors are not constitutively associated with Ptc.

Experiments in *Drosophila* (Yao et al. 2006, Zheng et al. 2010) and mammalian cell culture (Martinelli & Fan 2007) show that coreceptors synergize with Ptc to promote Hh binding to cells. Ligand binding in the presence of both a coreceptor and Ptc is considerably greater than the sum of binding when only one is present; more strikingly, *Drosophila* Ptc does not bind ligand in the absence of Ihog (Yao et al. 2006). This synergistic binding activity requires both the Hh- and Ptc-binding domains of the coreceptor (Zheng et al. 2010), matching the requirements for coreceptor-mediated promotion of Hedgehog signaling in vivo (Song et al. 2015, Tenzen et al. 2006, Zheng et al. 2010) or in vitro (Bae et al. 2011). However, the mechanism underlying coreceptor-mediated binding of Hh to Ptc remains subject to debate. One complicating issue is that Ihog and Boi are required for proper surface presentation of Ptc (Zheng et al. 2010). In the absence of affinity measurements, it is thus unclear whether the observed synergistic binding represents an increase in affinity, as expected for a stable heteromeric coreceptor–Ptc complex, or an increase in the number of available binding sites, as expected if coreceptors promote Ptc localization to the cell surface.

Available data on the interaction between coreceptors, Hh, and Ptc suggest that different coreceptors have different mechanisms for promoting Hh signaling. Ihog/Boi (Beachy et al. 2010; McLellan et al. 2006, 2008) and Gas1 (Martinelli & Fan 2009) bind Hh through sites that do not overlap with the ligand's putative Ptc-binding site, consistent with formation of a ternary complex. In contrast, Cdon and Boc bind a site on Hh that overlaps with that bound by Ptc, and the FnIII domain of Cdon competes with Ptc for Hh binding (McLellan et al. 2008). These observations suggest that Cdon and Boc may compete for ligand with Ptc rather than directly facilitate Hh–Ptc binding. Thus, Cdon/Boc may facilitate Hh–Ptc binding indirectly, concentrating Hh on the membrane of receiving cells and thus increasing the likelihood of its productive interaction with Ptc. Consistent with such a mechanism, a chimeric coreceptor consisting of the first and second FnIII repeats of Cdon and the third FnIII repeat of Boc does not bind Ptc and yet promotes ectopic signaling in vivo (Song et al. 2015), raising the intriguing possibility that IgSF coreceptor function may be independent of physical association with Ptc.

Due to cell-autonomous and non-cell-autonomous effects, Hh-binding proteins like Ptc and the coreceptors affect Hh signaling in vivo in complex ways by affecting the distribution and

FEEDBACK IN HEDGEHOG SIGNALING

Transcriptional feedback controls are characteristic features of many signaling pathways. In the case of the Hedgehog pathway, most feedback control mechanisms are negative, limiting the magnitude of the evoked response. A number of positive regulators of the pathway [such as the vertebrate coreceptors Cdon, Boc (Tenzen et al. 2006), and Gas1 (Allen et al. 2007)] are transcriptionally inhibited by Hh stimulation; conversely, the negative regulators Ptc1 (Capdevila et al. 1994, Marigo & Tabin 1996), Ptc2 (Motoyama et al. 1998b, Rahnama et al. 2004), and Hhip (Chuang & McMahon 1999) are upregulated by signaling. Interestingly, all these feedback-regulated proteins bind Hh. Thus, this feedback regulates the sensitivity of responding cells to ligand and shapes the Hh morphogen gradient (Chen & Struhl 1996, Gallet & Therond 2005, Holtz et al. 2013, Torroja et al. 2004).

Although these feedbacks are known to have important roles in Hh-mediated patterning during embryogenesis, understanding their full effect will require quantitative study. Appropriate methods need to be developed to measure the distribution of Hh and that of its binding partners in time and space, as well as cellular responsiveness to Hh. Quantifying these parameters and then integrating them with the cellular movement characteristic of developing tissues are major challenges, yet such developments promise to yield key insights into the mechanisms of metazoan development and to provide clues to the evolution and diversity of metazoan body plans.

potency of Hh. This complexity is compounded by transcriptional feedback control of the expression of these proteins. For more information, see sidebar titled Feedback in Hedgehog Signaling.

THE ROLE OF PATCHED IN HEDGEHOG SIGNALING

Ptc inhibits Smo, and Hedgehog signaling is triggered when Hh binds and inhibits Ptc, thereby derepressing Smo. The biochemical basis for these inhibitory interactions remains poorly understood. We discuss some recent findings pertaining to these processes.

Patched Repression of Smoothed

Initial models for Ptc function proposed that it inhibits Smo through direct binding and that ligand-induced disassociation of the complex results in Smo derepression (Goodrich & Scott 1998, Murone et al. 1999, Stone et al. 1996). This model was largely abandoned after it was found that Ptc and Smo have distinct subcellular distributions (Corbit et al. 2005, Deneff et al. 2000, Huangfu & Anderson 2005, Rohatgi et al. 2007). In the absence of Hh, Ptc is distributed over the entire plasma membrane in *Drosophila* cells or is concentrated in the primary cilium in vertebrate cells. In unstimulated cells, Smo is degraded (in the case of *Drosophila* Smo) or is sequestered in an endomembrane compartment (in the case of vertebrate Smo). Upon pathway stimulation by Hh, Ptc is endocytosed and degraded, leading to Smo accumulation over the entirety of the plasma membrane (*Drosophila*) or just in cilia (vertebrates).

Currently, it is thought that Ptc acts as an ion-driven pump to inhibit Smo activity, perhaps by transporting a metabolite. This model is consistent with two observations. First, Ptc shares some similarity with bacterial RND permeases, which use proton gradients across the plasma membrane to pump toxic substances out of cells (Li & Nikaido 2009, Tseng et al. 1999). Second, small amounts of Ptc are sufficient to inhibit Smo, suggesting that Ptc acts in a catalytic manner (Ingham et al. 2000, Taipale et al. 2002).

Ptc and bacterial RND proteins share a conserved topology, with 12 transmembrane helices (TMs) and an extracellular domain consisting of two large loops (loop 1 between TM1 and TM2 and loop 2 between TM6 and TM7). Although the primary sequence of Ptc and RND proteins diverges substantially, TM4 harbors a conserved GxxxDD motif, where x symbolizes amino acids A/V/G/L/I. Crucially, the first aspartate residue of the motif is required for function in both Ptc and RND proteins (Su et al. 2006, Taipale et al. 2002, Yu et al. 2003). In the case of RND pumps, the aspartate is part of a proton relay network. Protonation/deprotonation of the aspartate side chain is coupled to conformational changes in the rest of the protein such that substrates are bound or released, coupling proton conductance to substrate pumping (Pos 2009, Seeger et al. 2006). Bacterial RND permeases are trimers, and Ptc also appears to form oligomers. This is indicated by the observations that Ptc monomers coimmunoprecipitate with each other (Lu et al. 2006) and that loss-of-function Ptc mutants act as dominant negatives toward wild-type Ptc (Johnson et al. 2000, Martín et al. 2001).

The hypothesis that Ptc functions as an ion-driven small-molecule pump to inhibit Smo is attractive because it is also consistent with the idea of Smo regulation by an endogenous metabolite. It has been proposed that, in vertebrates, Ptc uses the energy of a proton gradient to expel vitamin D3 or provitamin D3 from cells and that vitamin D3 or provitamin D3 would then bind and inhibit Smo (Bijlsma et al. 2006, Roberts et al. 2016). Although it is formally possible that Ptc regulates Smo by supplying it with an inhibitor, this model conflicts with a number of observations. First, there is no proton-motive force across the plasma membrane of vertebrate cells. Second, other reports did not confirm inhibition of vertebrate Smo by vitamin D3 or provitamin D3 (Sever et al. 2016, Wilson et al. 2009). Third, vertebrate Smo appears to require a small-molecule activator rather than an inhibitor (Cooper et al. 2003).

Another model, based on studies in flies, proposes that *Drosophila* Ptc inhibits *Drosophila* Smo by regulating trafficking of lipoprotein-derived lipids (Callejo et al. 2008, Eaton 2008, Khaliullina et al. 2009, Panáková et al. 2005). Specifically, Ptc would mediate the export of a lipid factor that is necessary for inhibition and/or degradation of Smo. This model is consistent with the observation that, in *Drosophila*, Smo levels are low in the presence of Ptc and rapidly increase upon pathway stimulation (Denef et al. 2000, Zhu et al. 2003). The model is also consistent with the fact that the C-terminal tail of *Drosophila* Ptc, which is necessary for its endocytosis (Johnson et al. 2000, Lu et al. 2006), is also important for *Drosophila* Smo repression. A key prediction of this model is that endocytosis of Ptc is necessary for Smo repression; however, blocking endocytosis does not result in pathway activation (Torroja et al. 2004). Also significant is that modulating lipoprotein levels affects *Drosophila* Smo levels but is accompanied by only a modest effect on Hedgehog target gene expression (Callejo et al. 2008, Khaliullina et al. 2009, Panáková et al. 2005).

On the whole, the mechanism by which Ptc represses Smo remains unclear. Any proposed model would have to account for the observed conservation between Ptc and RND permeases and for the high potency of Ptc as a Smo repressor. Additionally, a model for Ptc should be consistent with established mechanisms of Smo regulation, such as the key role of the CRD of Smo and its associated ligand(s) (see section titled Activation of Smoothened). The mechanism of Smo regulation by Ptc may differ between invertebrates and vertebrates. One indication of divergence is the differential requirement of the cytoplasmic tail of Ptc, which is required for pathway repression in *Drosophila* but is dispensable in vertebrates (Harvey et al. 2014, Johnson et al. 2000). Some conserved residues are also differentially required for activity: D584 in *Drosophila* Ptc is necessary for pathway repression, whereas the homologous D585 in mouse Ptc1 is dispensable (Johnson et al. 2002).

Inhibition of Patched by Hedgehog Ligand

Soon after the *hh* and *ptc* genes were identified and cloned (Goodrich et al. 1996, Hooper & Scott 1989, Ingham et al. 1991, Lee et al. 1992, Nakano et al. 1989, Roelink et al. 1994, Tabata & Kornberg 1994), it was discovered that Hh directly binds the extracellular domain of Ptc with low-nanomolar affinity (Briscoe et al. 2001, Chen & Struhl 1996, Fuse et al. 1999, Marigo et al. 1996, Pepinsky et al. 2000, Stone et al. 1996). The Ptc-binding surface of Hh is thought to overlap with the surface that binds Cdon/Boc, Hhip, and the inhibitory antibody 5E1 (Beachy et al. 2010, Maun et al. 2010). Binding to Ptc requires the globular part of Hh (residues around the pseudoactive zinc hydrolase site) and a stretch of residues located within a 22-amino-acid extended N-terminal peptide of Hh (Fuse et al. 1999, Hall et al. 1995, Pepinsky et al. 2000, Tukachinsky et al. 2016, Williams et al. 1999).

Palmitoylation of Hh is critical for Ptc inhibition, as shown by experiments in which palmitoylation was blocked by genetic inactivation of Hhat/Ski (Chamoun et al. 2001, Konitsiotis et al. 2014), by pharmacological inhibition of Hhat (Petrova et al. 2013), or by mutation of the acceptor cysteine in Hh (Goetz et al. 2006, Pepinsky et al. 1998). Consistent with these observations, unpalmitoylated Shh is much less potent than palmitoylated Shh (Dawber et al. 2005, Kohtz et al. 2001, Lee et al. 2001). Additionally, a Shh mutant lacking palmitoylation and the first nine amino acid residues (Shh Δ 9) is completely inactive. Strikingly, Shh Δ 9 binds Ptc with the same high affinity as does palmitoylated Shh and, moreover, acts as a dominant-negative inhibitor toward the latter (Williams et al. 1999). This finding indicates that the palmitoylated N-terminal peptide of Shh is required for Ptc inhibition at a step downstream of high-affinity binding.

Recently, a short, palmitoylated N-terminal peptide of Shh was shown to be sufficient for Ptc inhibition and Hedgehog pathway activation (Tukachinsky et al. 2016). The peptide inhibits Ptc by direct binding, including a contact between the palmitoyl moiety and Ptc. This interaction is separable from the high-affinity binding of Ptc to the rest of Shh, indicating a two-pronged contact between Shh and Ptc, reminiscent of the interaction observed between palmitoylated Wnt and Frizzled (Fz) (Janda et al. 2012). Interestingly, although the palmitoylated peptide mimics Shh by inhibiting Ptc, it differs from Shh in its effect on Ptc trafficking and turnover. Shh induces Ptc endocytosis (Incardona et al. 2000) and degradation, whereas the palmitoylated peptide does not (Tukachinsky et al. 2016). Thus, internalization and inhibition of Ptc are separable, and internalization is dispensable for Ptc inhibition. This conclusion is consistent with *in vivo* experiments that show that Ptc endocytosis is not required for pathway activation by Hh (Torroja et al. 2004). Interestingly, Shh Δ 9 induces Ptc internalization, but not pathway activation, suggesting that internalization alone is not sufficient for inhibition; perhaps internalization is not complete, and Ptc left on the cell surface is sufficient for Smo repression.

The fact that Ptc bound to the palmitoylated peptide is inactive but not internalized suggests that it may adopt a conformation distinct from unliganded Ptc (which is active and on the cell surface) and from Shh-bound Ptc (which is inactive and internalized). Furthermore, the palmitoylated peptide does not bind point mutants of Ptc that cause Gorlin cancer syndrome and that have greatly reduced activity; this observation is consistent with these disease mutants adopting a conformation different from that of wild-type Ptc. Together, these results suggest the possibility that Ptc, like RND permeases, undergoes conformational cycling as part of its Smo-repressing function. In this model, inhibition of Ptc by Hh or by oncogenic mutations would result from conformational trapping, which interrupts normal cycling.

Significantly, there are contexts in which palmitoylation of Hh is not absolutely required for pathway activation, such as in some tissue explant experiments or *in vivo* (Kohtz et al. 2001, Lee et al. 2001, Roelink et al. 1995); this dispensability may be due to the higher sensitivity of some

tissues to Hh. For example, in tissues expressing less Ptc, ligand-induced internalization, which does not require ligand palmitoylation, may lower surface Ptc levels enough to trigger Hh pathway activation.

In contrast to *Drosophila*, which has one Ptc gene, vertebrate genomes have two orthologs: Ptc1 and Ptc2 (Carpenter et al. 1998, Motoyama et al. 1998b). The two proteins are very similar in sequence and behave similarly with respect to inhibition of Smo and response to Hh (Alfaro et al. 2014, Holtz et al. 2013, Klein et al. 2016, Lee et al. 2006, Zhulyn et al. 2015). However, Ptc1 is clearly the major regulator of Smo in vivo, as Ptc2 seems to be less potent and is expressed at much lower levels than Ptc1 (Motoyama et al. 1998a,b).

ACTIVATION OF SMOOTHENED

Smo belongs to class F/class 6 of the seven-transmembrane (7TM) superfamily of receptors, which includes Fz proteins. This class contains an N-terminal CRD, which, in the case of Fz proteins, binds Wnt ligands. It has long been thought that Smo is regulated by an endogenous ligand, like other 7TM receptors. Consistent with the existence of an endogenous ligand, vertebrate Smo harbors at least two separable small-molecule-binding sites: one in the CRD and the other in the 7TM domain. A number of crystal structures of Smo homologs were recently published: some of the isolated CRD (Huang et al. 2016, Nachtergaele et al. 2013, Rana et al. 2013), some of the 7TM portion of the protein (Wang et al. 2013, 2014; Weierstall et al. 2014), and one of the CRD together with the 7TM domain (Byrne et al. 2016). These structures have shed light on ligand binding and recognition by the two sites in Smo and on the nature of the endogenous ligand.

The Smo CRD fold is similar to that of the Frizzled 8 (Fz8) CRD. Both CRDs are stabilized by five disulfide bridges, and both feature homologous hydrophobic binding grooves that serve as ligand-binding sites. In the case of the Fz8 CRD, the groove is the binding site for the palmitoyl moiety of Wnt (Janda et al. 2012), whereas in the Smo CRD, the site binds sterols [such as cholesterol and 20(*S*)-hydroxycholesterol] or sterol-like molecules (such as cyclopamine and 22-azacholesterol) (Byrne et al. 2016, Huang et al. 2016, Nachtergaele et al. 2013, Nedelcu et al. 2013).

The transmembrane helices of Smo pack in a manner similar to that of class A/class 1 GPCRs. The 7TM domain of vertebrate Smo harbors a second small-molecule-binding site, which can be occupied by a large number of structurally diverse antagonists and agonists. Notable examples of Smo antagonists that bind the 7TM site are SANT-1, cyclopamine, and vismodegib, the last finding application in the clinic as an anticancer drug. An example of a 7TM-binding agonist is the synthetic small-molecule SAG (Chen et al. 2002). Although crystal structures of the Smo 7TM domain in complex with agonists and antagonists have been solved (Byrne et al. 2016, Wang & Pernow 2002, Wang et al. 2013), it is not yet clear how these small molecules modulate Smo, as no large conformational changes have been observed between agonist- and antagonist-bound structures.

For vertebrate Smo, several lines of evidence suggest that the endogenous ligand is likely an activating sterol. First, sterols are necessary for Smo activation, as shown by nonspecific sterol depletion and by inhibition of cholesterol biosynthesis (Cooper et al. 2003). Second, some oxysterols, such as 20(*S*)-hydroxycholesterol, activate Hh signaling at the level of Smo (Corcoran & Scott 2006, Dwyer et al. 2007). Activation of Smo by sterols is mediated by their interaction with the CRD (Nachtergaele et al. 2013, Nedelcu et al. 2013), and sterol binding to the CRD correlates well with activation of the Hedgehog pathway. Finally, blocking sterol-CRD binding by mutations in Smo (Byrne et al. 2016, Huang et al. 2016, Nachtergaele et al. 2013, Nedelcu et al. 2013), or pharmacologically (Nedelcu et al. 2013), inhibits Smo activation by Hh.

Cyclopamine:

a natural steroidal alkaloid antagonist of Smo; named for its cyclopia-causing teratogenic effect

SANT-1: a synthetic Smo antagonist

Vismodegib:

a synthetic Smo antagonist developed by Genentech for treatment of basal cell carcinoma; the first FDA-approved Hedgehog pathway antagonist

SAG: a synthetic Smo agonist

Although oxysterols, and particularly 20(S)-hydroxycholesterol, can activate Smo, they are present in cells at much lower levels than is necessary for activation (Myers et al. 2013), making them unlikely candidates for endogenous ligands. Recent studies indicate that cholesterol is the endogenous activating ligand for vertebrate Smo. Cholesterol binds the Smo CRD and activates Hedgehog signaling (Byrne et al. 2016, Huang et al. 2016); the binding constant for the Smo CRD–cholesterol interaction is well within the range of endogenous cholesterol levels. Furthermore, cholesterol, but not oxysterols, synergizes with Hh to activate Smo and Hedgehog signaling (Huang et al. 2016). Finally, CRD mutants of Smo, which are resistant to activation by oxysterols, remain sensitive to activation by cholesterol and to Hh (Huang et al. 2016, Luchetti et al. 2016).

An interesting question is whether the 7TM ligand-binding site of Smo is involved in Hedgehog signaling, particularly because no endogenous ligands are known to bind to it. Mutants of the 7TM site of Smo that completely abolish binding of several different synthetic ligands (both agonists and antagonists) still respond to Hh and to sterols (Huang et al. 2016). Although a hypothetical endogenous ligand might bind to the mutated 7TM site, these results suggest that the 7TM site likely does not participate in Smo regulation during normal Hedgehog signaling. The 7TM site, despite its doubtful physiological role, has proven very useful for developing highly potent small-molecule inhibitors of Smo.

The case for a ligand for *Drosophila* Smo is less clear than for vertebrate Smo. Although the CRD of *Drosophila* Smo is necessary for Hedgehog signaling (Aanstad et al. 2009), there is currently no sterol that is known to be necessary and/or sufficient for activation. Glucocorticoids were proposed to bind the CRD of *Drosophila* Smo (Rana et al. 2013); if so, functional experiments will be needed to determine whether this class of molecules modulates *Drosophila* Smo activity. Alternatively, the activating ligand for *Drosophila* Smo may not be a sterol, or else the protein may not require a small-molecule ligand for its activation.

SUMMARY POINTS

1. The Hedgehog ligand is modified with both palmitoyl and cholesteryl moieties, causing it to attach strongly to membranes.
2. The Hedgehog ligand requires the membrane protein Dispatched and extracellular chaperones for release from producing cells and for long-range signaling.
3. The Hedgehog ligand binds heparan sulfate proteoglycans, which are required for its extracellular movement.
4. Extracellular distribution of the Hedgehog ligand is negatively regulated by its binding to its membrane receptor, Patched, and to the secreted antagonist Hhip.
5. In the absence of ligand, Patched inhibits Hedgehog signaling by repressing the downstream seven-transmembrane protein Smoothened.
6. The Hedgehog ligand activates signaling by binding and inhibiting Patched, thus derepressing Smoothened.
7. The coreceptors Cdon/Ihog, Boc/Boi, and Gas1 cooperate with Patched for ligand reception and are collectively necessary for Hedgehog pathway activation.
8. Smoothened is activated by an endogenous ligand via its extracellular domain; in vertebrates, the Smoothened ligand is cholesterol.

FUTURE ISSUES

1. What is the mechanism of Dispatched-catalyzed Hedgehog ligand release?
2. What is the role of various factors proposed to function as extracellular chaperones for the Hedgehog ligand? To what extent are different chaperones unique to model systems or signaling contexts? How do these chaperones interface with downstream factors involved in Hedgehog ligand transport and reception?
3. How can we employ cell culture, genetic, and biochemical approaches to study movement of Hedgehog ligands in a more tractable, in vitro system?
4. How do Hedgehog coreceptors cooperate with Patched to facilitate ligand reception? Do coreceptors perform redundant or unique roles?
5. What molecular features distinguish Hedgehog coreceptors from Hedgehog antagonists? What determines whether a Hedgehog-binding protein potentiates or inhibits signaling?
6. How does Patched inhibit Smoothed? What are the substrate and source of energy for Patched?
7. How is the Smoothed-cholesterol interaction regulated?
8. How does Smoothed couple to downstream Hedgehog pathway activation?

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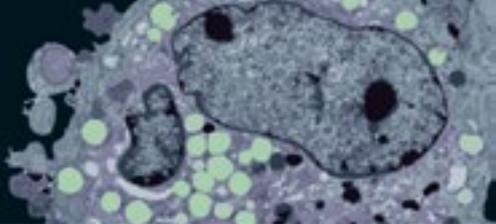
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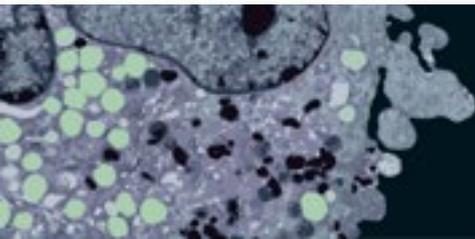
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