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The Inseparable Relationship Between Cholesterol and Hedgehog Signaling

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Abstract

Ligands of the Hedgehog (HH) pathway are paracrine signaling molecules that coordinate tissue development in metazoans. A remarkable feature of HH signaling is the repeated use of cholesterol in steps spanning ligand biogenesis, secretion, dispersal, and reception on target cells. A cholesterol molecule covalently attached to HH ligands is used as a molecular baton by transfer proteins to guide their secretion, spread, and reception. On target cells, a signaling circuit composed of a cholesterol transporter and sensor regulates transmission of HH signals across the plasma membrane to the cytoplasm. The repeated use of cholesterol in signaling supports the view that the HH pathway likely evolved by coopting ancient systems to regulate the abundance or organization of sterol-like lipids in membranes.

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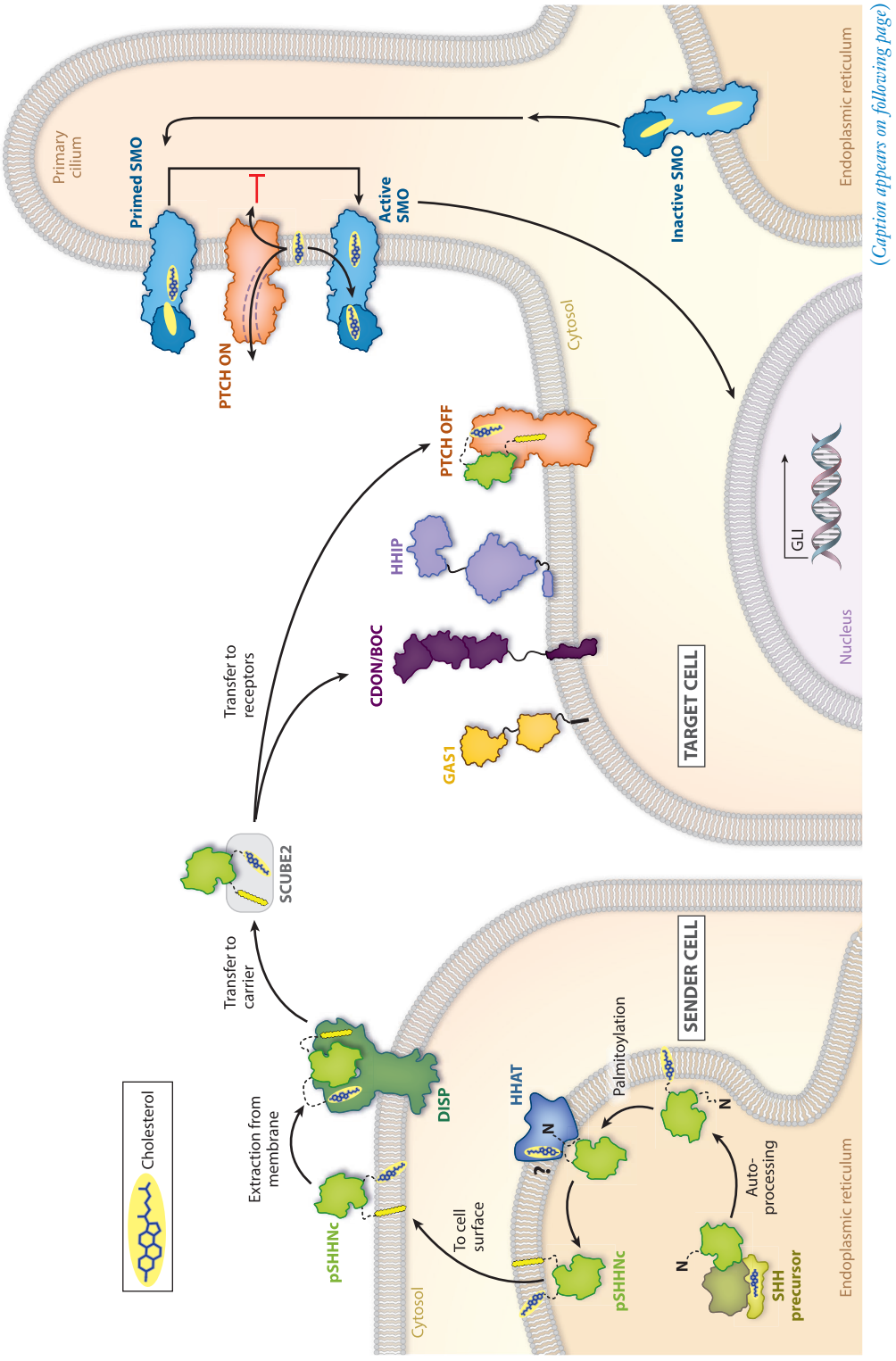
INTRODUCTION

The Hedgehog (HH) pathway (reviewed in 1, 2) is one of a handful of signaling systems that regulate early development and adult tissue homeostasis in most multicellular animals. Deployed in myriad ways, it can regulate cell proliferation, cell fate, patterning, and morphogenesis (3). Impaired HH signaling causes birth defects affecting many organs and drives cancers of the skin and brain. Mechanistic studies of HH signaling over the past three decades have uncovered multiple seemingly unrelated regulatory roles for cholesterol, distinct from its established role as a structural lipid in animal membranes. Cholesterol is required both for the biogenesis of mature HH ligands secreted by signal-sending (sender) cells and for transduction of the signal across the plasma membrane (PM) in signal-receiving (target) cells (**Figure 1**). We describe the function of cholesterol in both ligand secretion and reception and review recent work illuminating how proteins involved in these steps function as cholesterol transporters, transfer agents, and sensors to enable transmission of the HH signal from the sender cell to the cytoplasm of the target cell.

OVERVIEW OF HEDGEHOG SIGNALING

HH ligands are best known for their function as morphogens. They spread across tissues (as far as several hundred micrometers) to influence proliferation, cell fate determination, and patterning. There is one gene encoding a HH ligand (Hh) in insects like *Drosophila* and varying numbers of genes in different vertebrate species. For example, mammals have three ligands: Sonic Hedgehog (SHH), Indian Hedgehog (IHH), and Desert Hedgehog (DHH). HH ligands act on target cells through an unusual double-negative regulatory motif (4). The main receptor for HH ligands is a 12-pass transmembrane (TM) protein called Patched (abbreviated PTCH to include Patched in *Drosophila* and both Patched 1 and Patched 2 in vertebrates). PTCH is active in the absence of HH ligands and actively suppresses signaling by inhibiting Smoothened (SMO), a 7-pass TM protein of the G protein–coupled receptor (GPCR) superfamily. When SMO is inactive, HH-specific transcription factors (Ci in *Drosophila* or GLI in vertebrates) are converted to transcriptional



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Summary of HH signal transmission from sender to target cells highlighting the multiple roles of cholesterol (*yellow oval*) in ligand biogenesis, secretion, and reception. HH pathway proteins (consistent across all figures) are drawn to scale by tracing around the solvent-accessible surface of structures or structural models. In sender cells, cholesterol is used in an autoproteolytic reaction that leaves cholesterol attached to the N-terminal signaling domain of HH ligands (HhN or SHHN). Cholesterol-modified HH ligands are further lipidated by attachment of a palmitate moiety to the amino terminus. These attached lipids increase the hydrophobicity of HH ligands and anchor them to the extracellular leaflet of the plasma membrane, requiring a transporter (DISP) and carrier (SCUBE2) for efficient release. On target cells, a signaling module composed of a cholesterol transporter (PTCH) and cholesterol sensor (SMO) regulates signal reception. PTCH reduces cholesterol accessibility in the membrane of primary cilium, keeping SMO off. HH ligands bind and inactivate PTCH by inserting their lipidic appendages into its extracellular domain. The consequent rise in local membrane cholesterol accessibility is sensed by SMO, which transduces the HH signal across the plasma membrane and ultimately activates the HH gene expression program through the GLI transcription factors. HH ligand reception by PTCH is regulated by coreceptors (CDON/BOC, GAS1) and decoys (HHIP). Abbreviations: BOC, brother of CDON; CDON, cell adhesion molecule-related/downregulated by oncogenes; DISP, Dispatched; ER, endoplasmic reticulum; GAS1, growth arrest specific protein 1; GLI, GLI family zinc finger; HH, Hedgehog; HHAT, Hedgehog acyltransferase; HHIP, Hedgehog-interacting protein; pSHHNc, palmitate-and-cholesterol-modified SHH; PTCH, Patched; SCUBE2, signal peptide, CUB domain and EGF-like domain containinvvg 2; SHH, Sonic Hedgehog; SMO, Smoothened.

repressors that suppress target genes. HH ligands bind and inactivate PTCH, allowing SMO to adopt an active conformation. Activated SMO initiates a cytoplasmic signaling cascade that promotes the activation of the Ci/GLI transcription factors and ultimately the execution of a context-specific HH gene expression program (**Figure 1**).

An enigmatic feature of HH signaling is that in vertebrates (but not in *Drosophila*), it depends on primary cilia (5). Primary cilia are solitary antenna-like cell surface projections composed of a microtubule-based axoneme enveloped by a membrane. This ciliary membrane is contiguous with the PM but has a distinct (and dynamic) membrane and protein composition. Primary cilia are ubiquitous organelles that play important roles in the detection and interpretation of various signals, including those between cells that regulate tissue development and homeostasis. Many HH pathway components, including PTCH and SMO, are localized in cilia, and HH ligand exposure leads to changes in the ciliary trafficking of these proteins (**Figure 1**) (6, 7). Thus, primary cilia can be considered compartments where HH signals are transmitted across the membrane and propagated to the GLI transcription factors.

THE ORGANIZATION OF CHOLESTEROL IN MEMBRANES

We begin by summarizing some fundamental physicochemical properties of cholesterol relevant for understanding how it functions in HH signaling (and other cell biological processes). Cholesterol is an amphiphilic lipid containing a fused tetracyclic steroid ring connected to a hydrophobic iso-octyl group on one end and a hydrophilic hydroxyl group at the other end (**Figure 2*a***). Cholesterol has vanishingly low solubility in aqueous media as a monomer. The critical micelle concentration of cholesterol in water has been measured to be 20–40 nM (8, 9). Beyond this concentration, cholesterol forms micelles driven by strong noncovalent bonds between aggregated monomers (with a free energy of micellization of ∼12 kcal/mol), presenting a significant barrier to its interactions with proteins. The combination of hydrophobicity and tendency to form stable micelles in aqueous media has important consequences for proteins that handle or sense cholesterol. First, proteins mostly sense cholesterol in membranes, rather than sensing cholesterol released from membranes. Second, the diffusional transfer of cholesterol through aqueous media is unlikely to be significant at timescales relevant for signaling. The half-life for cholesterol transfer between two membranes is in the order of hours. The slow step in spontaneous transfer (activation energy of ∼20 kcal/mol) is the desorption of cholesterol from the membrane into the aqueous phase (10). Consequently, cholesterol movement between membranes and proteins

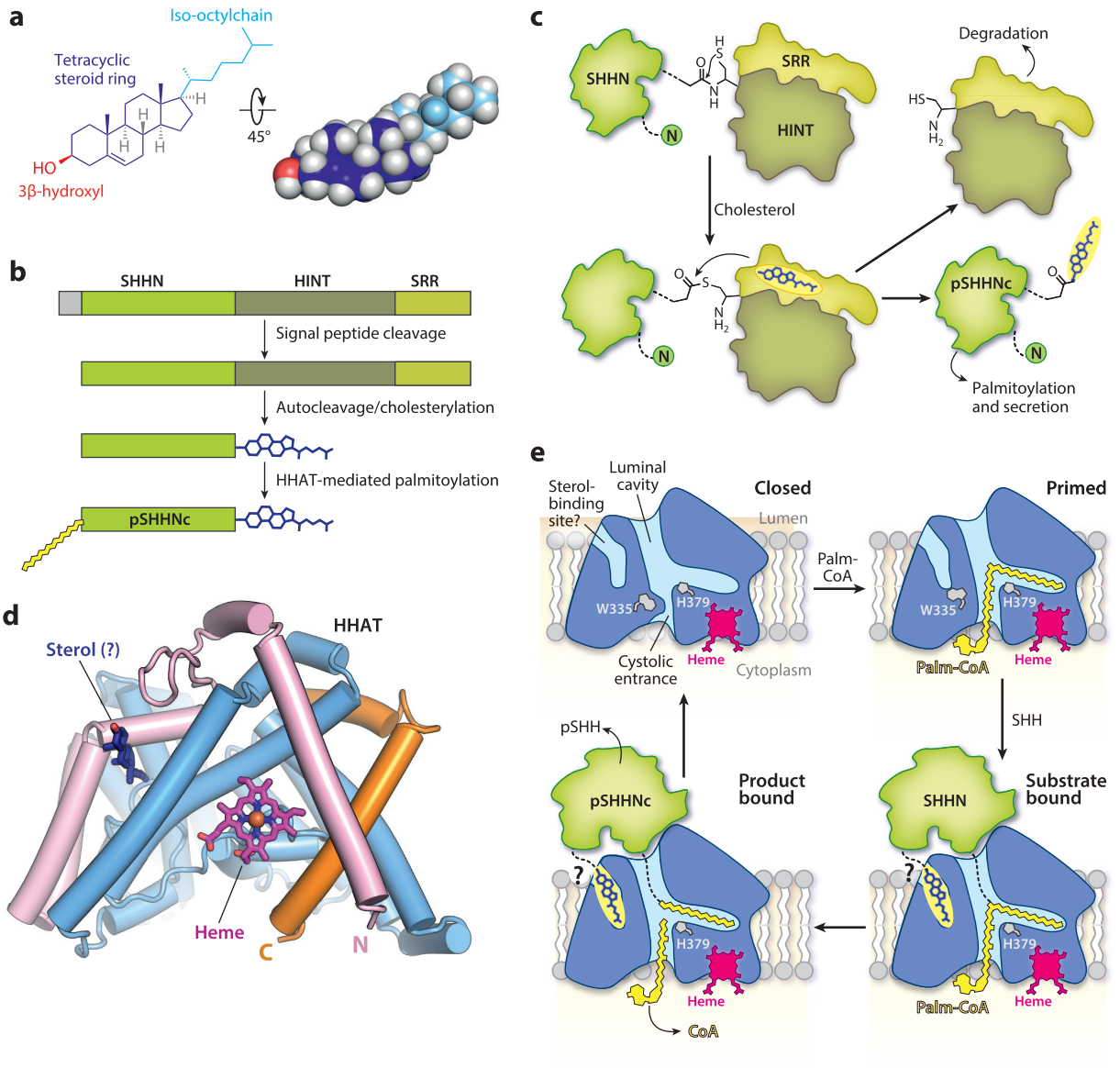


Figure 2

Biogenesis of HH ligands. (*a*) The atomic structure and space-filling model of cholesterol highlighting its amphiphilic nature. The 3β-hydroxyl (*red*) is hydrophilic, and the steroid ring (*dark blue*) and iso-octyl chain (*light blue*) are hydrophobic. Hydrogen atoms are depicted in gray. (*b*) The sequence of reactions that produce mature HH ligands covalently modified by a palmitate and cholesterol (pHhNc or pSHHNc). (*c*) A model for how cholesterol bound by the SRR functions as a nucleophile in an intein-like intramolecular cleavage reaction catalyzed by the HINT domain. (*d*) Cryo–electron microscopy structure of HHAT (PDB ID: 7Q6Z), the enzyme that catalyzes addition of a palmitate group to the N terminus of SHH. Note the bound sterol and heme groups. (*e*) A model for the reaction cycle of HHAT, which binds its two substrates, SHHNc and palmitoyl-CoA, on opposite sides of the ER membrane. The active site histidine and gate keeper tryptophan are highlighted in gray stick representation. Abbreviations: CoA, coenzyme A; ER, endoplasmic reticulum; HH, Hedgehog; HHAT, Hedgehog acyltransferase; HINT, hedgehog-intein; palm-coA, palmitoyl-coA; PDB ID, Protein Data Bank identifier; pSHH, palmitate-modified SHH; pSHHNc, palmitate-and-cholesterol-modified SHH; SHH, Sonic Hedgehog; SHHN, N-terminal domain of SHH; SHHNc, cholesterol-modified SHH; SRR, sterol-recognition domain.

or between two proteins involves a direct hand-off mechanism involving close donor–acceptor contact that allows transfer without requiring exposure to the aqueous phase. One example is the transfer of cholesterol from the Niemann-Pick C2 (NPC2) protein to the N-terminal domain of the Niemann-Pick C1 (NPC1) protein, allowing cholesterol to move from the lumen to the limiting membrane of the lysosome (11). Cholesterol transfer between membranes is thought to occur at membrane contact sites, where proteins like oxysterol-binding protein shield cholesterol during transport between two closely opposed membrane leaflets (12). In summary, specialized protein machineries are often required to overcome the activation energy barrier for cholesterol extraction from membranes.

Cholesterol is an abundant lipid in vertebrates, but its distribution in various cellular membranes is not uniform. There is a gradient of cholesterol abundance, with the lowest levels (5 mole% of lipids) found in the endoplasmic reticulum (ER) and the highest levels (>30 mole% of lipids) in the PM and its endocytic derivatives (13, 14). Several decades of work on membrane structure has revealed that PM cholesterol exists in at least two pools that differ in chemical activity (reviewed in 15–17). Chemical activity is a measure of the effective concentration of a species, which can be different from the actual concentration in nonideal environments (like membranes) due to interactions with other components. Since the chemical activity of cholesterol is difficult to measure directly in the PM, it is usually inferred indirectly from the accessibility of cholesterol to enzymes like cholesterol oxidase or soluble acceptors like methyl-β-cyclodextrin (MβCD). The majority (>95%) of cholesterol molecules in the PM are sequestered in complexes with membrane phospholipids and thus have low accessibility. Sphingomyelin (SM), an abundant lipid in the outer leaflet of the PM, forms particularly strong complexes with cholesterol and suppresses its activity (18). The smaller pool of cholesterol (1–5%) with high chemical accessibility plays a major role in regulating many membrane-dependent events, including signaling through the sterol regulatory element-binding protein cleavage-activating protein (SCAP)/sterol regulatory element-binding protein (SREBP) pathway that regulates cholesterol homeostasis (15), cell–cell spread of *Listeria* (19), and cellular entry of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (20). This body of work suggests that the abundance of accessible (not just total) cholesterol is an important parameter when considering cholesterol–protein transactions.

BIOGENESIS OF HEDGEHOG LIGANDS

Mature HH ligands are dually lipidated: They are covalently attached to a palmitate at the N terminus and cholesterol at the C terminus through two independent reactions in the ER (**Figure 2*b*,*c***). HH ligands are synthesized as precursors that carry a signal sequence for translocation into the ER. This precursor is composed of a N-terminal signaling domain (HhN in *Drosophila* and SHHN, IHHN, or DHHN in vertebrates) (21) followed by a catalytic hedgehog-intein (HINT) domain with homology to self-splicing inteins (22) and a C-terminal sterol-recognition domain (SRR) (**Figure 2*b***). The HINT domain catalyzes the intramolecular attack of an internal cysteine on the backbone carbonyl of the previous glycine, resulting in an N-to-S acyl transfer that leaves the C-terminal HINT–SRR module attached to HhN through a labile thioester bond (23, 24) (**Figure 2*c***). In a second step, the SRR facilitates the attack of the 3β-hydroxyl of a cholesterol molecule on this thioester, leaving the cholesterol molecule covalently attached to the C terminus of HhN (25, 26). The addition of cholesterol is hereafter denoted as HhNc or SHHNc.

A remaining question is how cholesterol from the ER membrane is recruited for the second step to serve as the nucleophile for the cleavage reaction. This is a challenge, since the thioester between the HhN and HINT–SRR domains can be cleaved by other nucleophiles, including water. Since cholesterol likely does not diffuse into the aqueous ER lumen to participate in the processing of

HH ligands, two possibilities are that (*a*) the SRR picks up a cholesterol from the luminal leaflet of the ER (like a sterol transfer protein) and positions it in a shielded way for thioester attack or (*b*) the SRR promotes the association of HH ligands with the ER luminal leaflet so cholesterol can attack the thioester without having to complete desorb from the membrane (27). A second consideration is that cholesterol abundance in the ER is thought to be the lowest in the cells (<5 mole% of total lipids), raising the question of how the SRR gains access to cholesterol (13). Interestingly, even though the total cholesterol content of the ER is low, the abundance of accessible cholesterol in the ER is comparable to that in the PM and thus may be adequate to drive HH ligand processing (28).

In addition to cholesteroylation, SHH also carries a palmitoyl adduct at its N terminal, a second hydrophobic modification that increases its potency in cell culture assays of HH signaling (29, 30). The palmitoylated ligand is referred to as pHhN or pSHHN and the dually lipidated ligand as pHhNc or pSHHNc. Palmitoylation is carried out in the ER by the membrane-bound O-acyltransferase family protein Hedgehog acyltransferase (HHAT), which belongs to the acyltransferase subclass EC 2.3.1 (31, 32). Recent structures of HHAT solved using cryo–electron microscopy (cryo-EM) show how this TM protein brings together its two substrates, palmitoyl-CoA (palm-CoA) and SHHN, from opposite sides of the ER membrane to catalyze the acyl transfer reaction (**Figure 2*d***) (33, 34). HHAT is composed of water-accessible luminal and cytosolic pockets that bind to SHHN and palm-CoA, respectively, and converge toward the catalytic reaction center (**Figure 2*e***). A potential sterol-binding site was observed in close proximity to the entry of the luminal SHHN access tunnel (**Figure 2*d***), suggesting that the cholesterol moiety attached to SHHN could enhance its recruitment to HHAT (**Figure 2*e***). Surprisingly, there is a heme group bound to a cysteine residue located in the HHAT cytosolic pocket that is essential for function, suggesting an additional level of regulation (**Figure 2*d***).

Cholesteroylation of HH ligands renders them tightly anchored to membranes (35, 36), initially in the lumen of the ER and subsequently on the outer leaflet of the PM after their transport to the cell surface through the secretory pathway (**Figure 1**). Studies in *Drosophila* have shown that membrane-attached HH ligands (HhN fused to a TM domain or other membrane anchor) can mediate short-range signaling between adjacent cells (37, 38) but cannot signal at a distance, a cardinal property of these morphogens. HH ligands can signal over 8–10 cell diameters in *Drosophila* (39) and up to 30 cell diameters in mammalian tissues (40), which requires dedicated mechanisms for the extraction of lipidated HH ligands from the PM of sending cells and shielding of their attached lipid appendages during transport through the aqueous interstitial tissues until they engage their primary receptor, PTCH.

SECRETION AND SPREAD OF HEDGEHOG LIGANDS

The extraction of cholesterol-modified HH ligands from the PM of sender cells is mediated by the protein Dispatched (DISP) (38, 41–43). DISP function is required selectively in sender cells that secrete HH ligands but not in target cells that respond to HH ligands. DISP function is essential for long-range signaling by HH ligands (but not for juxtacrine signaling between adjacent cells), identifying DISP as part of a mechanism dedicated to HH ligand release. DISP is required specifically for the release of the cholesterol-modified form of HhN: Mutant versions that cannot be cholesteroylated are released normally from cells lacking DISP (38). The cholesterol adduct attached to SHH can be photo-crosslinked to DISP in cultured cells, providing evidence for a physical interaction (36).

The cloning of the *disp* gene in *Drosophila* revealed its homology to both PTCH and NPC1, a transporter that mediates the export of cholesterol from lysosomes (38, 44, 45). All three proteins are members of the large resistance-nodulation-division (RND) family of transporters found

across the tree of life in archaea, eubacteria, and eukaryotes (46, 47). RND family proteins in bacteria are efflux pumps that use the energy of TM proton gradients for active transport. PTCH, NPC1, and DISP also share a sterol-sensing domain (SSD), a module composed of five TM helices that is found in many proteins that sense or transport cholesterol (48). Several lines of evidence show that DISP functions as a bona fide transporter to release cholesteroylated SHH from cells. First, DISP expression in cultured cells increases the release of SHH into the media (41), and the rate of SHH release increases with increasing DISP concentration (49). Second, a set of acidic residues at the center of the TM helices of DISP are required for its function in release of cholesteroylated SHH (41, 49). These residues are partially conserved in RND transporters, where they mediate the coupling of proton transport to ordered conformational changes that drive efflux. The transport activity of DISP is dependent on the ubiquitous TM sodium (Na^+) gradient across the PM, suggesting that it functions as a Na^+-driven antiporter for cholesteroylated SHH release from cells (49). Finally, several cryo-EM structures of DISP confirm the structural similarities between DISP, PTCH, NPC1, and the broader RND family, including residues putatively involved in interactions with monovalent cations (50–53). The structures provide insight into how DISP accommodates a protein substrate for transport (**Figure 3**). Cleavage of the first DISP extracellular domain (ECD1) by the proprotein convertase furin at an evolutionarily conserved site is required for DISP function (54). This cleavage reaction allows the ECDs of DISP to adopt a splayed arrangement to accommodate HH ligands. Models based on these structures propose that DISP extracts a cholesteroylated SHH from the outer leaflet of the membrane and then releases it to an extracellular carrier with conformational movements driven by changes in Na^+ occupancy at conserved binding sites in the center of the TM domain (36, 49, 50) (**Figure 3**).

As might be expected from the hydrophobicity of cholesterol, DISP does not release HH ligands into the aqueous interstitial space but rather transfers them to a carrier that can shield their

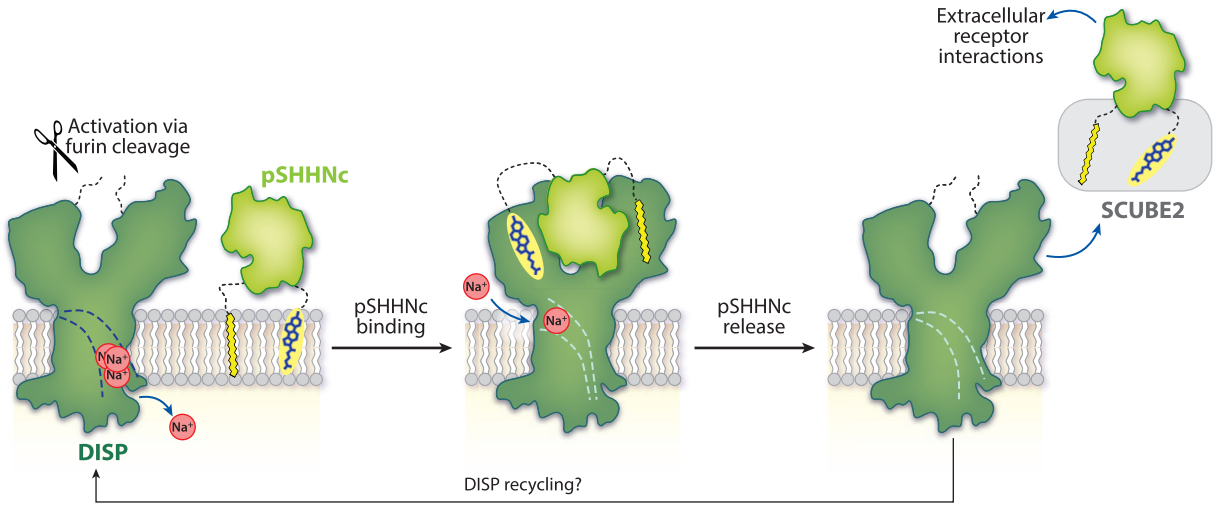


Figure 3

A model for the function of the DISP transporter powered by the electrochemical gradient of sodium (Na^+) across the membrane. DISP extracts pSHHNc from the outer leaflet of the plasma membrane and transfers it to the carrier SCUBE2 using a series of poorly understood conformational changes driven by the movement of sodium (Na^+) through its transmembrane domain. A furin-mediated cleavage of the DISP extracellular domain allows accommodation of the protein portion of pSHHNc. Abbreviations: DISP, Dispatched; pSHHNc, palmitate-and-cholesterol-modified Sonic Hedgehog; SCUBE2, signal peptide, CUB and EGF-like domain-containing protein 2.

lipidic appendages (**Figure 3**). In vertebrates, the best understood carrier is SCUBE2, a secreted protein first implicated in zebrafish as a non-cell-autonomous component that promotes HH signaling (55–57). The disruption of all three *scube* genes leads to a near-complete loss of HH signaling in zebrafish embryos (58). SCUBE2 binds to the lipidic appendages of SHH and is required for the release of SHH by DISP in cultured cells (36, 59, 60). The SHH–SCUBE2 complex described in these studies is likely the same as a diffusible, higher molecular weight (~100-kDa) form of SHH described a decade earlier that mediated long-range signaling (61). Crosslinking experiments suggest that the cholesterol appendage of SHH is handed off from DISP to SCUBE2, perhaps guiding the directional transfer of the ligand (36). Genetic analysis of the three *scube* genes in mice is complicated by redundancy, so it is not known whether SCUBE proteins are required universally for HH ligand release from sending cells in the mammalian embryo. Importantly, SCUBE orthologs are not found in *Drosophila*, suggesting that other acceptors can fulfill this function, such as the product of the *shifted* gene (62, 63).

As morphogens that pattern a tissue field, HH ligands secreted from sender cells have to spread over significant distances through tissues. A large body of active research, primarily in *Drosophila* but also in vertebrate systems, has identified several mechanisms for the spread of HH ligands: extracellular vesicles, cytonemes, lipoprotein particles, and heparan sulfate proteoglycans (64–69). Due to space constraints, we do not discuss these mechanisms in detail but refer readers to recent reviews (1, 70). Each of these mechanisms provides a potential solution to the problem of shielding the lipidic appendages of HH ligands during their journey through tissues.

HEDGEHOG LIGAND RECEPTION ON TARGET CELLS BY PATCHED

HH ligands bind and inactivate PTCH, allowing the activation of SMO and transmission of the HH signal to the cytoplasm (71, 72). In addition to inhibiting SMO, PTCH also reduces the abundance of HH ligands by promoting their endocytosis and lysosomal degradation (73). PTCH functions as a central node in a negative feedback loop that limits the magnitude, duration, and spatial distribution of HH signaling: HH signaling activates PTCH mRNA transcription, and the resulting increase in PTCH protein both inhibits SMO activity and depletes extracellular HH ligands. The three HH ligands in mammals (SHH, DHH, and IHH) are expressed in different tissues but bind to PTCH and coreceptors with similar affinities and activate signaling with similar efficacies. Most of the biochemical analysis has focused on SHH, but we expect these mechanisms to be conserved in IHH and DHH as well.

Structural studies reveal an unusual tripartite interaction between pSHHNc (dually lipidated SHH) and PTCH (**Figure 4*a*,*b***). Two molecules of PTCH (74, 75) bind to one molecule of pSHHNc in a 2:1 asymmetric complex. One molecule of PTCH engages the lipid moieties attached to SHH (the lipid interface). The N-terminal palmitate (76) and the C-terminal cholesterol (77, 78) of pSHHNc are both bound in a PTCH intramolecular tunnel that starts at the top of the ECD and ends at the outer leaflet of the PM. The same pSHHNc molecule uses its opposite face to make a high affinity protein–protein interaction (the protein interface) with a second PTCH molecule (**Figure 4*b***). The tunnel in PTCH that accommodates the two lipid adducts of SHH has been proposed to be the conduit for cholesterol transport, suggesting a model for how HH ligands block its biochemical activity (74–76, 78–81).

Early studies of pSHHN designed to assess the functional consequences of palmitoylation revealed a disparity between PTCH affinity and signaling potency in cultured cell assays. SHHN bereft of both its cholesterol and palmitoyl moieties bound PTCH with roughly the same (low nanomolar) affinity as pSHHN (29, 30). However, pSHHN was nearly 50-fold more potent at inhibiting the biochemical activity of PTCH (and consequently activating the transcription of

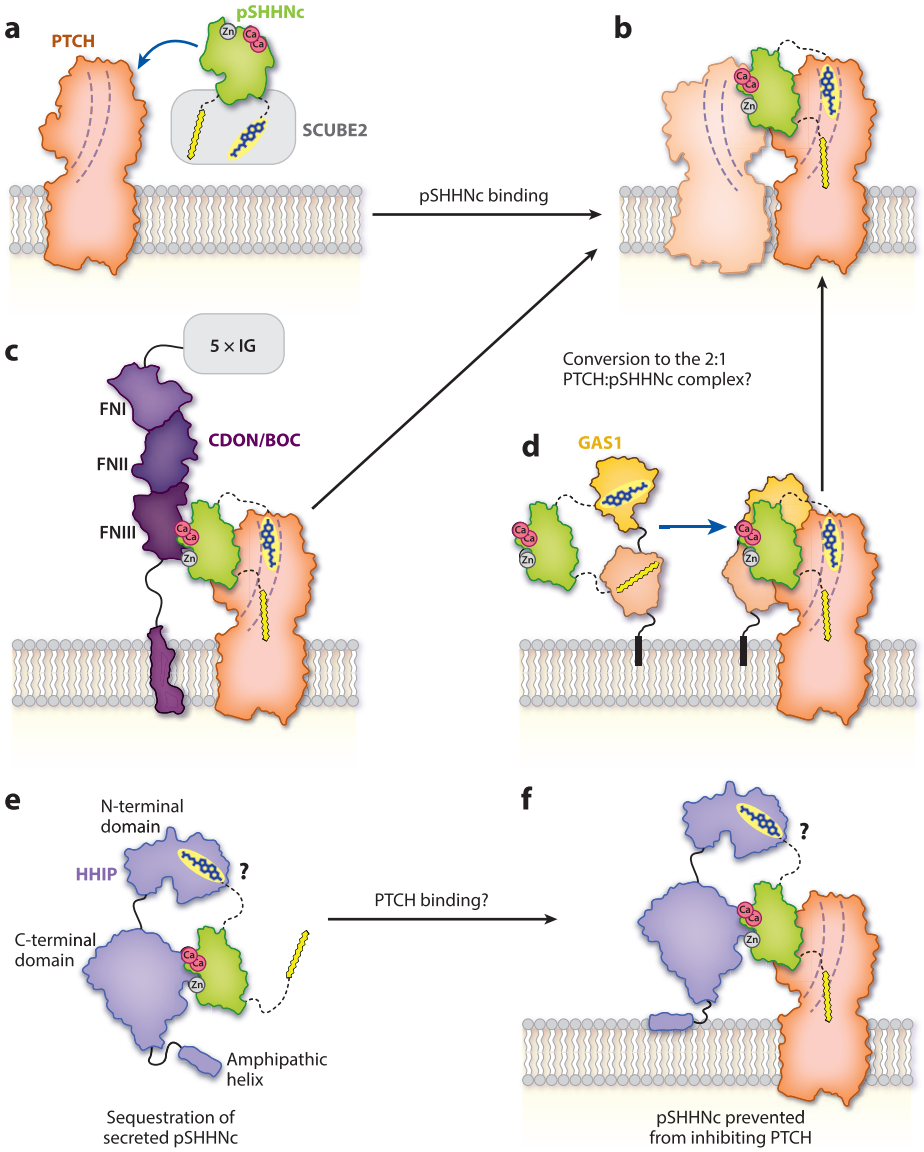


Figure 4

Coreceptors and decoys regulate the reception of HH ligands by PTCH. (*a*,*b*) pSHHNc binds two PTCH molecules simultaneously using different interfaces: The blue PTCH engages pSHHNc at a protein interface that includes its calcium- and zinc-binding sites, and the green PTCH binds to the N-terminal palmitoyl and C-terminal cholesterol modifications of the same pSHHNc molecule. The dotted line outlines an intramolecular tunnel in PTCH that may serve as a conduit for cholesterol transport and is obstructed by insertion of the lipidic pincers from pSHHNc. Assembly of the SCUBE2–pSHHNc complex is depicted in **Figure 1**. (*c*,*d*) Models for how coreceptors CDON/BOC and GAS1 can promote the binding of pSHHNc to PTCH. (*e*,*f*) HHIP inhibits pSHHNc, even though it does not block its binding to PTCH. Abbreviations: BOC, brother of CDON; CDON, cell adhesion molecule-related/downregulated by oncogenes; FN, fibronectin repeats; GAS1, growth arrest-specific protein 1; HH, Hedgehog; HHIP, Hedgehog-interacting-protein; IG, immunoglobulin repeats; pSHHNc, palmitate-and-cholesterol modified Sonic Hedgehog; PTCH, Patched; SCUBE2, signal peptide, CUB and EGF-like domain-containing protein 2.

HH target genes). A palmitoylated 22-amino-acid peptide of SHH is sufficient to trigger HH signaling at micromolar concentrations (82). The N-terminal palmitoylation of SHHN can be partially substituted by a variety of other hydrophobic modifications (such as installation of two isoleucine residues), suggesting that a linear straight-chain alkane is not strictly required to inactivate PTCH (30). In vivo data from *Drosophila* and mouse embryos also support a role for the palmitate modification in activating HH signaling in target cells (83, 84). Taken together, these studies show that the insertion of the attached palmitate into the PTCH ECD is important for receptor inactivation.

In cryo-EM structures of the pSHHNc–PTCH complex, the C-terminal cholesterol attached to SHH occupies a pocket at the apex of ECD1 of PTCH (77, 78). The isolated PTCH ECD1 can bind to polyethylene glycol–modified cholesterol in solution and has been crystallized in complex with cholesterol hemisuccinate (78). In vitro, the cholesteroylation of SHHN enhances its potency in signaling, supporting the structural data (78). SHHNc is also more active than SHHN when expressed from a transgene in the limb mesenchyme of mouse embryos (83). Several studies have tested the phenotypic consequences of eliminating the cholesterol modification of HH ligands in embryos, though the direction of the outcome is different in *Drosophila* and mice. In *Drosophila* embryos, eliminating the cholesterol modification in HhN overexpressed in the wing disc or in embryos expands the range of signaling (23, 38). In contrast, preventing cholesterol modification in mouse embryos restricts the range of signaling and the spread of SHHN in the limb bud (40). While it is difficult to cleanly distinguish between effects on ligand spread and PTCH inactivation in embryos, the data support a role for the cholesterol moiety in PTCH inactivation, especially at longer distances from the ligand source (40, 78). The cholesterol modification likely plays an additional role in the spread of HH ligands in mouse embryos by facilitating their interactions with carriers like SCUBE2, an effect that may be less relevant for the shorter distances that HH ligands have to travel in *Drosophila* (40).

It is important to remember that SHHN lacking all lipid modifications can also activate HH signaling when added to various culture systems at 50–100-fold higher concentrations than pSHHN or pSHHNc (29). This observation suggests that interaction of SHH with PTCH through the high-affinity protein interface can inactivate PTCH function in cells, either by triggering a conformational change (74) in PTCH or by inducing its endocytosis and consequent clearance from the plasma and ciliary membranes (82). The physiological reason why HH ligands have evolved to engage and inactivate PTCH through multiple, partially redundant interactions remains unknown. One possibility is that the protein-based interaction is most relevant for the function of PTCH in limiting the spread of HH ligands by sequestration, while the lipid-based interaction plays the dominant role in the biochemical inactivation of PTCH and transduction of HH signals to the cytoplasm (73).

CORECEPTORS AND DECOYS REGULATE THE RECEPTION OF HEDGEHOG LIGANDS

The reception of HH ligands by PTCH in target cells is regulated by several membrane-associated proteins that interact with PTCH and HH ligands. CDON and BOC, along with their *Drosophila* orthologs Ihog and Boi, are structurally related Type I single-pass TM proteins of the immunoglobulin superfamily identified as positive regulators of HH signaling (85–87) (**Figure 4*c***). A third positive regulator of HH signaling found only in vertebrates is GAS1, a glycosylphosphatidylinositol-linked protein (88) (**Figure 4*d***). Hedgehog-interacting protein (HHIP) is a vertebrate-specific SHH-binding protein that functions as an antagonist of HH signaling (89) (**Figure 4*e*,*f***). The transcription of genes encoding CDON, BOC, and GAS1 is suppressed by HH signaling in some contexts, while that of genes encoding HHIP and PTCH

is activated by HH signaling. Consequently, these function as components of negative feedback loops to limit HH responses in target cells.

Genetic studies in *Drosophila* and mice show that CDON, BOC, and GAS1 have overlapping, partially redundant functions. *Drosophila* lacking either Ihog or Boi are viable and fertile, but embryos (or imaginal disc clones) lacking both proteins are defective in their responses to HH (86). In mice, the abrogation of HH signaling requires the simultaneous disruption of *Cdon*, *Boc*, and *Gas1* (90, 91). Disrupting one of these genes has modest, tissue-specific effects on HH signaling, while disruption of two genes simultaneously has a phenotype of intermediate severity (92). CDON, BOC, Ihog, and Boi all bind to HH ligands through a protein-based interface adjacent to its metal ion–binding sites (86, 93, 94) (**Figure 4*c***). GAS1 (like SCUBE2) binds with high affinity to SHH through an interface that includes its cholesterol and palmitate appendages (60, 88, 95). There is evidence that CDON/BOC and Ihog/Boi associate with PTCH to form the functional receptor complex that receives HH ligands on target cells (91, 94) (**Figure 4*c***). In vertebrates, GAS1 associates with PTCH to form an alternative receptor complex distinct from the CDON/BOC–PTCH complex (91) (**Figure 4*d***).

Based on experiments in cultured cells, a sequential ligand relay mechanism has been proposed for how these coreceptors function as lipid transfer agents to catalyze the directional movement of lipidated HH ligands from SCUBE2 to PTCH (60, 95). First, CDON/BOC recruits the SCUBE–pSHHNc complex to the cell surface through protein-based interactions with both SCUBE2 and SHH. This recruitment step is followed by two ligand transfer steps driven largely by interactions with the lipid appendages of SHH: transfer of SHH from SCUBE2 to GAS1, followed by the transfer of SHH from GAS1 to PTCH. However, genetic data show that this GAS1-centric relay mechanism cannot be the only way in which SHH is received on target cells by PTCH, since it does not account for the redundancy between GAS1 and CDON/BOC noted in several systems (90, 91). In addition, the phenotype of $Gas1^{-/-}$ embryos is less severe than that of embryos lacking HH ligands, showing that HH ligands can activate signaling in target cells without the assistance of GAS1 (96–98). Finally, *Drosophila* lack GAS1 (and SCUBE2) but are still able to respond to dually lipidated HH ligands. The different modes by which HH ligands are transferred from carriers like SCUBE2 to PTCH may be operational at different ligand concentrations, different positions in the ligand gradient, or different developmental contexts. While multiple mechanisms may be used, a common principle is that HH coreceptors facilitate the transfer of lipidated HH ligands from carriers like SCUBE2 to PTCH, while shielding the attached cholesterol and palmitate groups from the aqueous interstitial medium.

HHIP is a glycoprotein tightly attached to cell-surface HSPGs that binds HH ligands and negatively regulates HH reception in target cells (89, 99). $Hhip^{-/-}$ embryos die shortly after birth with lung defects suggestive of enhanced HH signaling, but many other HH-dependent tissues such as the limb and the spinal cord develop normally. However, loss of HHIP in the background of a *Ptch1* allele that cannot be transcriptionally activated by HH ligands causes developmental defects consistent with an increase in the strength and extent of HH signaling (100). The C-terminal domain of HHIP tightly interacts with the metal-binding site found in HH ligands (101, 102) (**Figure 4*e***). Interestingly, HHIP also has an N-terminal cysteine-rich domain (CRD) that contributes to its inhibitory function, and this CRD has been proposed to bind to the cholesterol or palmitate appendages of HH ligands (103) (**Figure 4*e***). By multi-modal binding and sequestration of HH ligands, HHIP, in cooperation with PTCH, limits the spread of HH ligands and their action on target cells (100, 104, 105). The molecular mechanism by which HHIP blocks HH ligand reception on target cells by PTCH remains to be clarified.

In summary, the panel of cell surface proteins that regulate reception of HH ligands by target cells uses interactions with cholesterol and palmitate adducts attached to HH ligands to regulate

the strength of HH signaling in a tissue like the neural tube, facilitating the dose-dependent responses to HH ligands that are required for proper patterning.

TRANSMISSION OF HEDGEHOG SIGNALS ACROSS THE PLASMA MEMBRANE

PTCH binds to extracellular HH ligands, but conformational changes in SMO transmit the HH signal across the membrane. A decades-long mystery in HH pathway research has been the question of how PTCH inhibits SMO. Since PTCH and SMO do not physically interact, a small molecule second messenger has been assumed to mediate this interaction (106). Several clues to this puzzle have emerged over the last few decades and are relevant to the current models for this key step in signaling.

First, depletion of cholesterol in target cells attenuates the strength of signaling. Exposure to inhibitors of late steps in the cholesterol biosynthesis pathway during pregnancy can lead to holoprosencephaly, a birth defect also seen with genetic or pharmacological inhibition of HH signaling (107, 108). Human syndromes caused by loss-of-function mutations in enzymes that catalyze late steps in cholesterol biosynthesis (e.g., Smith–Lemli–Opitz Syndrome) are characterized by birth defects in tissues dependent on HH signaling during development (109, 110). Indeed, HH signaling in cultured cells is attenuated by loss-of-function mutations in these genes or by the direct depletion of cholesterol using MβCD (109).

Second, PTCH (like DISP) has sequence similarity to NPC1, which transports cholesterol from the lumen of the lysosome to the limiting lysosomal membrane for delivery to other destinations in the cell (44, 45). PTCH, NPC1, and DISP contain an SSD and are members of the RND superfamily of transporters. A transporter function for PTCH itself was suggested by the observations that (*a*) PTCH is capable of inhibiting SMO in a catalytic manner (106, 111, 112), (*b*) mutations in acidic residues in the TM domain important for transport by RND transporters also impair PTCH function (49, 106), and (*c*) PTCH can bind to cholesterol and efflux BODIPY-cholesterol from cells (113).

Third, sterol-like molecules can function as SMO ligands. A naturally occurring steroidal alkaloid, cyclopamine, binds and inhibits SMO (114, 115). In addition, oxygenated metabolites of cholesterol called oxysterols bind and activate SMO. Oxysterols carrying hydroxyl groups in the iso-octyl side-chain (called side-chain oxysterols) can activate HH signaling in cultured cells (6, 116, 117). Oxysterols were found to activate HH signaling by directly binding SMO, leading to the hypothesis that the elusive endogenous ligand for SMO may be a sterol (118).

Finally, PTCH in vertebrates inhibits SMO at primary cilia (**Figure 1**). PTCH is concentrated in a punctate pattern along the membrane of the primary cilium and in a membrane invagination around the base of primary cilia known as the ciliary pocket (6). HH ligands induce changes in the localization of PTCH and SMO: PTCH is inactivated and cleared from the cilium while SMO accumulates in the ciliary membrane (6, 7). Activated SMO engages the downstream signaling machinery to transmit the HH signal to the cytoplasm. These dynamic trafficking changes led to the proposal that PTCH inhibits SMO at primary cilia, but the biochemical basis for this compartmentalization is unknown (6). Surprisingly, despite the conservation of PTCH and SMO, HH signaling in *Drosophila* does not require primary cilia.

SMOOTHENED IS A CHOLESTEROL-RESPONSIVE PROTEIN

The first crystal structure of SMO that contained both its TM and extracellular domains revealed a cholesterol molecule bound in a hydrophobic groove in its extracellular CRD (119) (**Figure 5**). The position of this cholesterol-binding site, >10 Å above the membrane outer leaflet, was notable

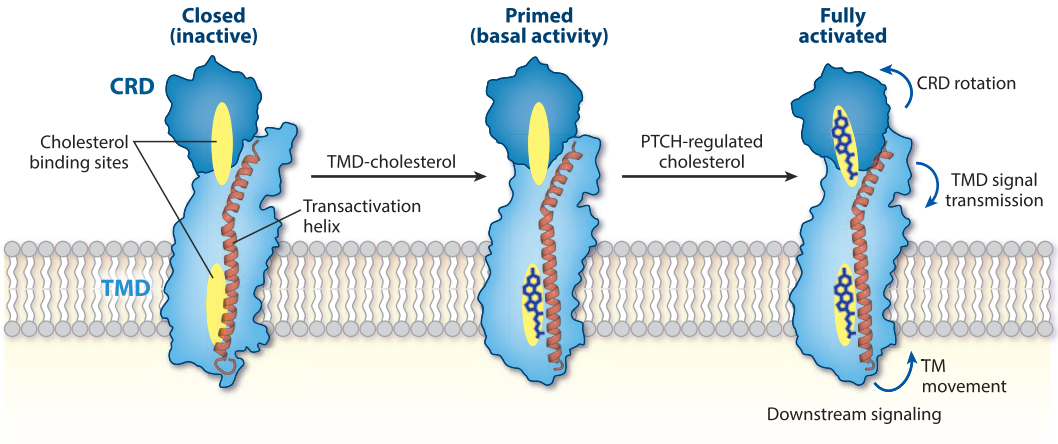


Figure 5

Activation of SMO by dual cholesterol binding to its TMD and its extracellular CRD. PTCH blocks SMO by preventing cholesterol binding to its CRD. Binding of cholesterol to the CRD causes it to rotate on its TMD pedestal. Signal transmission from the CRD to the cytoplasm is then facilitated by a long helix (*red*) that makes contacts with the CRD in the extracellular space and extends through the membrane to the cytoplasm. Outward movement of this helix is thought to allow SMO to activate cytoplasmic signaling components. Abbreviations: CRD, cysteine-rich domain; PTCH, Patched; SMO, Smoothened; TM, transmembrane; TMD, transmembrane domain.

because most cholesterol-binding sites in membrane proteins are located in the TMD. Oxysterols also bind and activate SMO through this same binding site (120–122). One group has suggested that cholesterol can be covalently attached to the SMO CRD, analogous to its attachment to HH ligands (123). More generally, evolutionary sequence and structural analyses suggest that the SMO CRD is derived from an ancient family of domains designed to bind and respond to hydrophobic small molecules (120, 124).

Cholesterol is both necessary and sufficient to activate SMO, providing functional evidence that cholesterol itself, in addition to oxysterols, can function as a SMO agonist (125, 126). Structure-guided mutations in the CRD that weaken cholesterol binding also impair responses to HH ligands in both cells and mouse embryos, showing that the structurally identified site in the SMO CRD is required for endogenous HH signaling (119, 123, 125, 126). Subsequent structural and functional studies found that SMO has at least one additional sterol-binding site located within the central cavity of its TMD (127–129) (**Figure 5**). Cryo-EM structures also suggest the presence of additional sterol-binding sites in an intramolecular tunnel that extends between the two crystallographically defined CRD and TMD sites in SMO, although the CRD is not well resolved in this structure (130).

A debated question in the field is which of these sterol-binding sites in SMO is regulated by PTCH to block HH signaling (the different views are articulated in 127, 128, 130, 131). Based on mutagenesis and sterol probes selective for the TMD and CRD sites, a model has been proposed that attempts to harmonize some of the seemingly conflicting findings (131) (**Figure 5**). Cholesterol plays two distinct roles in SMO activation. Cholesterol binding to the TMD site primes SMO, setting the magnitude of both its basal and stimulated signaling activity (131). However, the increase in SMO signaling activity when PTCH is inactivated by HH ligands is largely driven by cholesterol binding to the CRD site (131), which causes a small rotation of the CRD on its TMD pedestal (119) (**Figure 5**). Remarkably, the TMD and CRD sites seem to be allosterically

linked despite being >40 Å apart, making it difficult to disentangle the relative contributions of these sites to SMO regulation by PTCH (131). The observation that SMO activation required dual sterol binding is consistent with prior cell biological and pharmacological data showing that SMO activation in response to HH signaling is a two-step process (132). Interestingly, a cholesterol molecule would have to completely desorb from the membrane outer leaflet to bind to the CRD (125). Given the high energetic barrier to spontaneous desorption, how does cholesterol gain access to the CRD? One possibility, consistent with the observation of sterol-like cryo-EM densities along a channel that connects the TMD and CRD sites, is that cholesterol moves from the membrane to the TMD site and then up through the protein to bind to the CRD (130).

A second point of debate in the literature is whether the endogenous, PTCH-regulated ligand for SMO is cholesterol itself or an oxysterol that is produced from cholesterol (since both can activate signaling when added to cells) (125, 126, 129, 133). We favor the view that cholesterol itself is the main PTCH-regulated second messenger that controls SMO activity for two reasons. First, a separation-of-function mutation in the SMO CRD that maintains the response to cholesterol but abolishes the response to oxysterols does not impair signaling in response to HH ligands (which act on SMO indirectly through PTCH) (125, 126). Second, an unbiased loss-of-function clustered regularly interspaced short palindromic repeats (CRISPR) screen in cultured cells targeting a set of ∼1,200 lipid-related genes, including those involved in known sterol metabolic pathways, uncovered multiple enzymes at early and late steps in the pathway for cholesterol biosynthesis as positive regulators of HH signaling (134). No known oxysterol, steroid, or Vitamin D synthesis enzymes were identified as positive regulators, pointing to cholesterol itself, rather than a precursor or product, as the key sterol relevant for SMO activity. The requirement for 24(S), 25-epoxycholesterol, an oxysterol that can bind and activate SMO (129, 133), can be difficult to exclude since it is synthesized through a shunt pathway that closely parallels the postsqualene cholesterol biosynthesis pathway. Only one enzyme in the Kandutsch–Russell and Bloch pathways for cholesterol synthesis is dispensable for 24(S),25-epoxycholesterol synthesis: 24-dehydrocholesterol reductase (DHCR24). The observation that HH signaling is blocked in *Dhcr24*$^{-/-}$ cells [in the absence of any change in 24(S), 25-epoxycholesterol abundance] again supports a role for cholesterol in SMO activation (134).

An alternative model that should be considered is the possibility that SMO is regulated by a sterol antagonist (e.g., one that displaces cholesterol from SMO and is regulated by PTCH). Reduction of cellular levels of this putative antagonist would be expected to drive constitutive HH signaling, mimicking the loss of PTCH. However, both unbiased screens (134, 135) and directed experiments (110) have failed to identify genes in sterol synthesis or metabolism pathways that function as strong negative regulators of HH signaling.

SMOOTHENED IS A SENSOR OF ACCESSIBLE CHOLESTEROL IN MEMBRANES

In addition to being an abundant lipid, cholesterol regulates many processes in the cell, including the universal SCAP/SREBP signaling system that maintains cholesterol homeostasis by regulating both cholesterol biosynthesis and uptake (136). These considerations raise the question of how cholesterol can be used to regulate signaling through a pathway like HH that must be precisely controlled to avoid birth defects and cancer. Conversely, if HH ligands activate SMO by changing membrane cholesterol, there must be mechanisms that prevent undesirable crosstalk with other cholesterol-dependent processes. In the case of other second messengers like Ca^{2+} and cAMP that regulate multiple pathways, two general strategies are used to address the twin challenges of fidelity and specificity. First, the bulk cytoplasmic concentration is not the parameter most relevant

for signaling by many second messengers since their activities are held in check by protein binding or organelle sequestration (137). In this way, the free (or signaling-competent) pool is released only when needed. Second, spatial confinement in subcellular compartments or cytoplasmic nanodomains allows a potentially promiscuous second messenger like cAMP to activate only specific pathways with high fidelity in response to upstream inputs (138). Recent work has revealed that both principles are relevant for how cholesterol regulates SMO in the HH pathway.

SMO activation is driven by a minor pool of accessible cholesterol rather than by the total pool of cholesterol in the PM (134; for a recent in-depth discussion, see 139). Accessible cholesterol is the pool of cholesterol with higher chemical potential that is free of sequestration by SM and other membrane factors, analogous to the signaling-competent pool of Ca^{2+} or cAMP that is free from interactions with buffering proteins (137, 138). The same CRISPR screen that identified cholesterol biosynthesis enzymes as positive regulators of HH signaling also unexpectedly identified several genes required for the synthesis of SM, a lipid that had never been linked to HH signaling previously (134). In contrast to cholesterol genes, SM genes are negative regulators of HH signaling: SM depletion enhances the potency of HH ligands in both target gene transcription and differentiation assays in vitro. Since SM is the major phospholipid that sequesters cholesterol in the PM, its depletion increases the size of the accessible cholesterol pool and consequently enhances SMO activity (15). Experiments using pharmacological approaches and microbial toxin-derived probes to monitor and perturb the accessible pool of cholesterol showed that changes in cholesterol accessibility produce corresponding changes in HH signaling strength, even when the total membrane cholesterol concentration remains unchanged (134). In addition to SM depletion, disruption of *Canopy4*, a gene encoding a saposin-like protein, causes enhanced HH signaling at the level of SMO in cells and embryos due to increased accessible (rather than total) cholesterol in the plasma and ciliary membrane (140). These studies show that SMO functions as a sensor of cholesterol accessibility in membranes.

When HH ligands are applied to cells, changes in cholesterol accessibility are restricted to the membrane surrounding the primary cilium, the subcellular compartment where PTCH inhibits SMO and where HH signaling is orchestrated in vertebrates (134) (**Figure 1**). PM-accessible cholesterol remains unchanged, explaining why HH signaling does not alter overall cholesterol homeostasis in cells or interfere with other cholesterol-dependent processes. Thus, the enigmatic spatial confinement of HH signaling to primary cilia in vertebrates may have allowed this pathway to use the organization of cholesterol in membranes as a signaling messenger. The local increase in cholesterol accessibility in the ciliary membrane induced by HH ligands leads to cholesterol binding to the extracellular SMO CRD and ultimately to the conformational changes that drive transmission of the HH signal to the cytoplasm.

PTCH FUNCTIONS AS A TRANSPORTER TO REDUCE CHOLESTEROL ACCESSIBILITY IN MEMBRANES

If cholesterol is the endogenous second messenger that regulates HH signaling, a key prediction is that PTCH must be able to somehow prevent cholesterol from binding to SMO (**Figure 6**). Analogous to DISP, the homology of PTCH to NPC1 and the broader family of RND proteins, including the conservation of key acidic residues along the central channel of the TMD required for ion transport in RND proteins, is consistent with the hypothesis that PTCH is an active sterol transporter that uses the energy of a monovalent cation gradient (106). Multiple Cryo-EM structures of PTCH have provided circumstantial support for this model (74–76, 79, 81). These structures reveal sterol-like densities along a putative transport conduit that extends through the PTCH ECD and ends at the outer leaflet of the PM at the level of the conserved

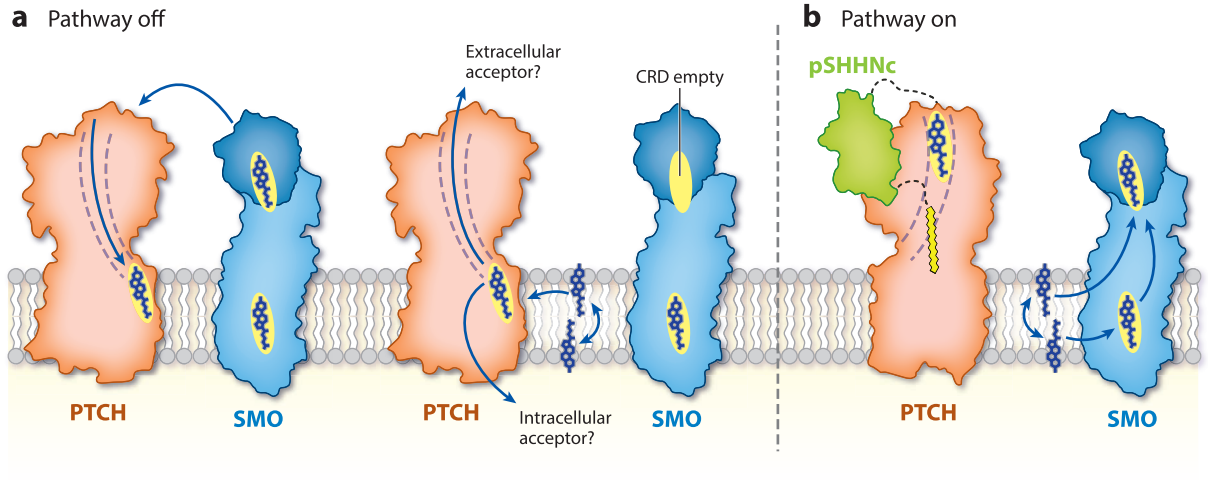


Figure 6

Models for how PTCH inhibits SMO. (*a*) PTCH can (*left*) directly remove the cholesterol molecule bound to the SMO CRD or (*right*) change the balance between inner and outer leaflet cholesterol by transporting cholesterol to an intracellular or extracellular acceptor. (*b*) Inactivation of PTCH by SHH causes cholesterol accessibility to rise in the membrane outer leaflet, allowing cholesterol to bind the CRD and activate SMO. Cholesterol has been proposed to move through an intramolecular tunnel in SMO to reach the CRD. The reactions depicted here are locally confined to the membrane of the primary cilium (**Figure 1**). Abbreviations: CRD, cysteine-rich domain; pSHHNc, palmitate-and-cholesterol-modified SHH; PTCH, Patched; SHH, Sonic Hedgehog; SMO, Smoothened; SSD, sterol-sensing domain.

SSD in PTCH (**Figure 6**). Mutations that disrupt this conduit impair the ability of PTCH to inhibit SMO. The sterol transport function of PTCH is likely to be ancient, predating even the evolution of HH signaling. PTCH has been proposed to have evolved from bacterial transporters for hopanoids, analogs of sterols in bacteria that are synthesized from squalene precursors (141).

Direct tests for whether PTCH can change membrane cholesterol composition have relied on fluorescently labeled protein probes derived from cholesterol-dependent cytolysins such as perfringolysin O (PFO) or anthrolysin O (142, 143). These studies have led to conflicting views of how PTCH changes the organization of cholesterol in membranes (**Figure 6*a***). Engineered versions of PFO-D4 (a domain of PFO that binds cholesterol but does not lyse cells) that detect total rather than accessible cholesterol led to the observation that PTCH overexpression in cultured cells reduced cholesterol abundance in the inner leaflet but increased it in the outer leaflet of the PM (79, 144). However, microbial and eukaryotic protein probes that detect accessible cholesterol selectively revealed that PTCH activity reduced outer leaflet cholesterol accessibility, including in the outer leaflet of the ciliary membrane (where SMO activity is regulated) (131, 134).

In an entirely different approach to understanding the biochemical activity of PTCH, a kinetic assay was developed based on the longstanding observation that the rate of cholesterol extraction from membranes by MβCD is a sensitive measure of cholesterol accessibility (17, 139, 145, 146). This property, which has been observed in synthetic monolayers and bilayers as well as in the PM of living cells (10), was foundational to the discovery that membrane cholesterol is segregated into accessible and sequestered pools. The rate of cholesterol extraction is determined by the activation energy for its transfer from the membrane outer leaflet to soluble acceptors like MβCD, which in turn is a direct reflection of its fugacity or chemical activity. This assay provided direct evidence that PTCH overexpression can reduce cholesterol accessibility in the outer leaflet of the PM (145).

Critically, this activity of PTCH was antagonized by its ligand SHH and by point mutations in acidic TMD residues thought to mediate monovalent cation transport. Given the localization of PTCH in the ciliary membrane in cells and embryos (6), this activity would reduce cholesterol accessibility in the outer leaflet of the ciliary membrane, preventing SMO activation (**Figure 6*a***). Inactivation of PTCH by HH ligands would lead to a rise in ciliary accessible cholesterol, which would activate SMO by binding to its CRD (**Figure 6*b***).

A third model for PTCH function discussed in the literature is the possibility that the ECD of PTCH directly removes cholesterol from the SMO CRD at primary cilia (2, 49, 111, 131) (**Figure 6*a***). Indirect evidence for this model comes from the observations that PTCH can reduce the crosslinking of both cholesterol (123) and oxysterol (131) analogs to the SMO CRD. This model is compatible with the one proposing that PTCH reduces cholesterol accessibility in the membrane outer leaflet. By reducing cholesterol accessibility in the outer leaflet, PTCH would increase the thermodynamic drive for the movement of cholesterol from the SMO CRD to the outer leaflet of the PM.

In general terms, a variety of assays converge on the idea that PTCH changes the organization of cholesterol in membranes, making it less accessible to soluble acceptors like MβCD, cholesterol-binding protein probes, and (by extension) to SMO. Given the rapid flip-flop of cholesterol between the two leaflets of the membrane, the seemingly different observations using inner and outer leaflet cholesterol probes may simply reflect an overall decrease in cholesterol accessibility. The model that PTCH blocks SMO activity by reducing membrane cholesterol accessibility requires that PTCH and SMO colocalize in the same membrane domain, one that is ideally insulated from bulk PM cholesterol fluxes that would tend to neutralize PTCH-mediated effects (**Figure 6**). We speculate that the ciliary membrane provides such a privileged compartment, where PTCH can locally deplete cholesterol accessibility without interfering with other processes in the cell. Indeed, the cilium represents one of the only places in the cell where PTCH and SMO colocalize (6, 132).

An instructive exception to the requirement of primary cilia for PTCH–SMO regulation is found in *Drosophila*. The origins of the sterol synthesis pathway, which makes cholesterol in animals, ergosterols in fungi, and phytosterols in plants, starting from a common squalene precursor, can be traced to the last common eukaryotic ancestor (147). However, nematodes and most arthropods are cholesterol auxotrophs, having lost the first three enzymes in the cholesterol biosynthesis pathway (148, 149). *Drosophila* membranes have much lower concentrations of cholesterol (<5%) (150), and the SCAP/SREBP system has been repurposed to regulate phospholipid synthesis (151). We speculate that loss of the connection between primary cilia and HH signaling in *Drosophila* may have been enabled by the lack of a need to insulate HH signaling from pathways that regulate cholesterol biosynthesis and uptake. This idea would benefit from investigation of whether cilia are required for HH signaling in other groups of multicellular invertebrates, a question that remains unresolved (152).

Despite the evidence summarized above, it is important to remember that the field still lacks a transport assay for PTCH in a completely purified system, rightfully considered the gold standard to establish the substrate specificity of a transporter protein and to elucidate mechanistic aspects such as the identity of the monovalent cation gradient that powers transport or the directionality of transport. Such an assay system can provide a definitive test of the hypothesis that cholesterol is the substrate for PTCH and also help distinguish various models of PTCH–SMO regulation.

EVOLUTION OF THE HEDGEHOG PATHWAY

An interesting duality is that cholesterol is both a substrate of PTCH and, when attached to HH ligands, an inhibitor (78). This observation supports an insightful model for HH pathway

evolution that may explain the many roles that cholesterol plays in the HH pathway, both in sender and in target cells (141). Notably, this model was proposed before much of the structural and biochemical work discussed in this review. HH signal sensing in target cells is driven by a pump–sensor circuit that monitors the cholesterol organization of a membrane domain: PTCH (the pump) uses its active transport function to reduce cholesterol accessibility, which in turn regulates the activity of SMO (the sensor). SMO activation induces transcription of the *Ptch* gene, which ultimately functions to constrain pathway activity by inactivating SMO. This type of circuit may have initially evolved to regulate the abundance of a membrane component like hopanoids (141). When the abundance of this component rises above its homeostatic level, it would activate the sensor, leading to transcription of the gene encoding the pump. Increased protein levels of the pump would then restore membrane composition, leading to inactivation of the sensor. With the advent of multicellularity, this circuit was coopted for cell–cell communication by the covalent linkage of the substrate for the pump (cholesterol) to a secreted ligand via an intein-mediated reaction. This ancestral HH ligand, secreted by sender cells, could now control the pump–sensor circuit in target cells by functioning as a substrate mimic that would sterically block pump function due to the attached protein. Modern HH ligands also use a palmitate appendage and a protein interface to inhibit PTCH function, but this model suggests that the ancestral mode of inhibition would have been through the attached cholesterol. We speculate that such pump–sensor modules may function in other contexts to control the organization of cholesterol in membrane domains to influence diverse membrane-dependent processes. The idea that PTCH and SMO were linked at some point in evolution (rather than coevolving) is supported by the fact that PTCH has ancestors that extend back to bacteria, while SMO-like proteins are found only in eukaryotes (141). Given these ideas, it would be revealing to test the role of cholesterol and other sterols in both PTCH–SMO and PTCH–HH ligand regulation across a broader range of metazoans, including cnidarians and lophotrochozoans, in which these components are conserved.

FUTURE ISSUES

1. We do not understand the molecular mechanism by which PTCH reduces membrane cholesterol accessibility. Important unresolved questions include the direction of cholesterol transport by PTCH and the identity of the cation gradient across mammalian cell membranes that provides the energy for PTCH function (49, 79, 145).
2. How does the ciliary membrane maintain a distinct lipid composition from the contiguous plasma membrane, and how can this composition be altered by signals to change the activities of cilia-localized proteins? Are there dedicated transporters, lipid transfer enzymes, lipid metabolic enzymes, or membrane contact sites that function at the ciliary pocket to regulate the composition of the ciliary membrane?
3. How does PTCH regulate SMO in *Drosophila* in the absence of a requirement for primary cilia and the absence of a cholesterol biosynthesis pathway? Does cholesterol or another sterol mediate the communication between PTCH and SMO, and is its availability regulated in a confined membrane domain or organelle? This answer will provide evolutionary insights and also help to clarify the core biochemical mechanism that mediates the inhibition of SMO by PTCH.
4. Are there other pump–sensor systems that regulate cholesterol accessibility locally to influence membrane-dependent events such as signaling, trafficking, synaptic transmission, and cell–cell contacts?

5. What are the precise functions of the various decoys and coreceptors that regulate reception of HH ligands by PTCH? How are their overlapping interactions with HH ligands integrated to control the precise strength of signaling delivered to target cells?

DISCLOSURE STATEMENT

R.R. and C.S. are consultants to Dark Blue Therapeutics.

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