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Authors
Nguyen, Thi D
Truong, Melissa E
Reiter, Jeremy F

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REVIEW
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The Intimate Connection Between Lipids and Hedgehog Signaling

Thi D. Nguyen[1†], *Melissa E. Truong*[2†] *and Jeremy F. Reiter*[1,3*]

[1]Department of Biochemistry and Biophysics, Cardiovascular Research Institute, University of California, San Francisco, San Francisco, CA, United States, [2]Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA, United States, [3]Chan Zuckerberg Biohub, San Francisco, CA, United States

Hedgehog (HH) signaling is an intercellular communication pathway involved in directing the development and homeostasis of metazoans. HH signaling depends on lipids that covalently modify HH proteins and participate in signal transduction downstream. In many animals, the HH pathway requires the primary cilium, an organelle with a specialized protein and lipid composition. Here, we review the intimate connection between HH signaling and lipids. We highlight how lipids in the primary cilium can create a specialized microenvironment to facilitate signaling, and how HH and components of the HH signal transduction pathway use lipids to communicate between cells.

Keywords: intercellular signaling, development, cholesterolyation, sterols, cilia

Edited by:
Matthias Lauth, Philipps University of Marburg, ZTI, Germany

Reviewed by:
Martin E. Fernandez-Zapico, Mayo Clinic, United States
Pascal Therond, CNRS UMR7277 Institut de Biologie Valrose, France

***Correspondence:**
Jeremy F. Reiter
jeremy.reiter@ucsf.edu

†These authors have contributed equally to this work

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INTRODUCTION

The HH pathway functions in metazoan development as one of the principal means of cell-cell communication (Ingham and McMahon, 2001; Ingham, 2018). HH was discovered in a *Drosophila* genetic screen for developmental regulators (Nüsslein-Volhard and Wieschaus, 1980). HH proteins are secreted ligands that are interpreted by receiving cells via the transmembrane proteins Patched (PTCH) and Smoothened (SMO) to control the activity of the downstream transcription factor effectors, called Cubitus interruptus in *Drosophila* and GLI in vertebrates (Nüsslein-Volhard and Wieschaus, 1980; Nüsslein-Volhard et al., 1984; Forbes et al., 1993; Quirk et al., 1997).

HH signaling is one fundamental mechanism by which cells communicate and is deployed both in development and adult physiology to control diverse tissue dynamics, including patterning and the regulation of cell growth. Consequently, defective HH signaling in development causes birth defects, and mis-activation of HH signaling postnatally can cause cancer.

As many HH pathway components are conserved between insects and vertebrates, it was unexpected when a genetic screen in mice identified proteins required for both vertebrate HH signaling and the formation of an organelle called the primary cilium (Huangfu et al., 2003). The primary cilium is a microtubule-based organelle found on most vertebrate cells (Wheatley, 1995; Wheatley et al., 1996). Unlike motile cilia, such as those found on cells in the airway, the brain ventricles, and the oviduct that beat to move overlying fluid, primary cilia are immotile and specialized for signal transduction (Ishikawa and Marshall, 2011).

The discovery that primary cilia are required to transduce mammalian HH signaling sparked investigation into the connection between HH signaling and the primary cilium (Bangs and Anderson, 2017). Research into primary cilia in diverse organisms has revealed that evolution has played with the role of cilia in transducing HH signals. Cilia are present in all clades of extant eukaryotes, indicating that they were probably present in the last eukaryotic common ancestor (LECA), whereas the HH pathway probably arose with multicellularity (**Figure 1**).

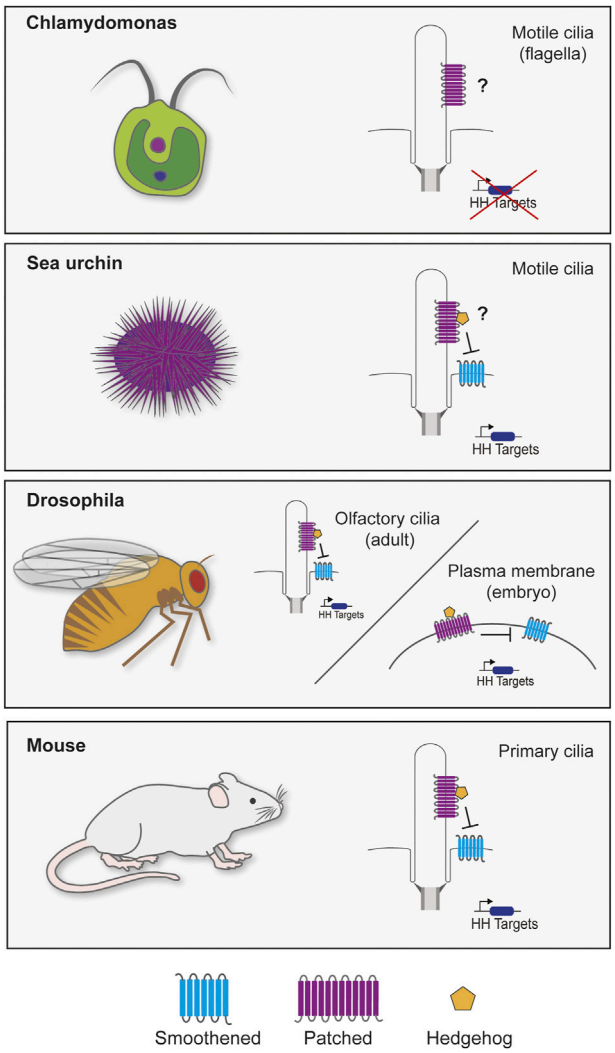

FIGURE 1 | Cilia and Hedgehog signaling throughout evolution. Though cilia are highly conserved, the reliance of HH signaling on the cilium varies through evolution. The green alga *Chlamydomonas reinhardtii* genome possesses two homologs of *PTCH* (Cre02.g093500 and Cre12.g496350), but not other components of the HH pathway. It will be interesting to determine whether either acts at the *Chlamydomonas* flagella. In sea urchin embryos, SMO localizes to cilia to activate HH signaling for mesoderm specification (Warner et al., 2014). Different tissues in *Drosophila* transduce HH signals with or without cilia. Most *Drosophila* cells lack cilia and transduce HH signals via Smo at the plasma membrane (Zhu et al., 2003; Jia et al., 2004). However, olfactory sensory neuron cilia in the adult fly brain signal through ciliary Smo (Kuzhandaivel et al., 2014). Vertebrates require primary cilia to transduce HH signals (Bangs and Anderson, 2017). Defects in ciliary transport or structure cause a wide range of HH-related phenotypes (Reiter and Leroux, 2017).

In vertebrates, coordinated protein trafficking of HH pathway components into and out of cilia is required for regulated signal transduction. In the absence of HH signals, Patched1 (PTCH1) and the G protein-coupled receptor GPR161 localize to the ciliary membrane (Rohatgi et al., 2007; Mukhopadhyay et al., 2013). Binding of a HH ligand, such as Sonic Hedgehog (SHH), to PTCH1 triggers exit of PTCH1 from the cilium which cues ciliary accumulation of SMO (Corbit et al., 2005). Once localized to the cilium, SMO converts GLI proteins, which localize to the ciliary tip, into transcriptional activators which leave the cilium, enter the nucleus, and induce HH target genes (Haycraft et al., 2005; Wen et al., 2010; Santos and Reiter, 2014).

One theoretical evolutionary advantage of scaffolding signal transduction within the primary cilium is that it may increase signaling fidelity by imposing an additional level of regulation through subcellular trafficking. Although the primary cilium shares a membrane that is contiguous with the plasma membrane, the cilium can signal distinctly from the rest of the cell (Delling et al., 2013; Marley et al., 2013; Truong et al., 2021). Key to its signaling functions is the maintenance of distinct ciliary protein and lipid compositions (Nachury, 2014; Mick et al., 2015).

Over the last decade, we have gained some understanding of how the protein composition of the cilium is controlled. For example, a region near the base of the cilium called the transition zone, recognized electron micrographically by prominent structures called Y-fibers connecting the axoneme to the ciliary membrane, controls protein accumulation within the cilium (Garcia-Gonzalo and Reiter, 2017; Nachury and Mick, 2019).

The distinct protein composition of the ciliary membrane raises the interesting question of whether the lipid composition of the ciliary membrane similarly differs from that of other cellular membranes. Less is understood about how different lipids are distributed throughout the cell, including at the cilium.

Broadly speaking, lipids play three biological functions: as energy storage, as the principal components of cellular membranes, and as participants in signal transduction. Lipid droplets store neutral lipids that can be catabolized to generate ATP. Cellular membranes are primarily composed of bilayers of amphipathic phospholipids. Other lipids, such as sterols and phosphoinositides, are non-uniformly distributed and define distinct cellular membranes. Subcellular differences in lipid composition affect membrane curvature, tension, and the function of signaling proteins (van Meer et al., 2008; Harayama and Riezman, 2018).

One intercellular communication pathway dependent on lipids is HH signaling. For example, lipidation of HH ligands is key to their activity and extracellular distribution as gradients to pattern developing tissues (Eaton, 2008). Downstream of HH, the HH receptor PTCH1 transports sterols to affect the composition of the membrane (Zhang et al., 2018; Kinnebrew et al., 2021; Qi et al., 2019). Sterols also regulate the activity of the central HH pathway component SMO (Cooper et al., 2003; Myers et al., 2013, 2017; Nachtergaele et al., 2013; Nedelcu et al., 2013; Byrne et al., 2016; Huang et al., 2016; Luchetti et al., 2016; Xiao et al., 2017; Huang et al., 2018; Raleigh et al., 2018). Still other lipids, phosphoinositides, are read out by TUBBY family proteins to control the trafficking of HH signal transduction component GPR161 to cilia (Chávez et al., 2015; Garcia-Gonzalo et al., 2015). In this review, we focus on the role of lipids in HH signaling, especially at the ciliary membrane. We examine how the lipid composition of the primary cilium creates a specialized microenvironment essential for vertebrate HH signaling. Additionally, we dissect how these lipids function in

embryonic development and how their dysregulation causes birth defects. Further research into how lipids function in HH signaling, particularly within the primary cilium, may illuminate general principles by which the subcellular distribution of lipids is controlled to contribute to protein function and the propagation of information.

CILIARY MEMBRANES HAVE A DISTINCT LIPID COMPOSITION

In protists, biochemical assessments have indicated that the lipid composition of cilia is distinct. For example, the ciliary membranes of *Paramecia* and *Tetrahymena* are enriched in phosphonolipids (consisting of the well-named ciliatine attached to a lipid backbone) and sphingolipids (Kennedy and Thompson, 1970; Smith et al., 1970; Andrews and Nelson, 1979; Kaneshiro et al., 1984). In *Paramecia*, a mutation that alters ciliary sphingolipid levels compromises the function of voltage-sensitive channels, suggesting that its distinct lipid composition is critical for ciliary protein function and that sphingolipids may be particularly important for ciliary biology (Forte et al., 1981).

One sphingolipid, sphingomyelin, can sequester sterols in complexes (Leathes, 1925; McConnell and Radhakrishnan, 2003; Das et al., 2014). Filipin, a mixture of polyene macrolides, binds 3-β-hydroxysterols and can be observed in freeze-fracture electron microscopy (Kinsky et al., 1966). In the distantly related protists Euglena and Trypanosomes, filipin staining revealed that sterols are enriched in the flagellar membrane (Melkonian et al., 1982; Souto-Padrón and de Souza, 1986; Tetley, 1986). In quail, filipin staining also demonstrated robust enrichment of 3-β-hydroxysterols in the ciliary membrane (Chailley and Boisvieux-Ulrich, 1985). Similarly, Laurdan staining of ordered lipids suggested that ciliary membranes are enriched in sterols (Tyler et al., 2009). As described further below, sterols contribute to HH signaling, and thus ciliary sphingolipids, by controlling the accessibility of sterols, can limit the signaling functions of the cilium. Indeed, sphingomyelin biosynthetic pathway enzymes restrain HH signaling (Kinnebrew et al., 2019).

How else might ciliary lipids contribute to ciliary protein function? One possibility is that they function as specific cofactors for ciliary proteins. Some lipids, such as phosphoinositides, may be at lower molar concentrations than their interacting proteins and thus may function as regulatory cofactors. Another possibility is that ciliary lipids impart a distinct biophysical or biochemical property to the ciliary membrane which is itself important for protein function. Lipids help determine membrane viscosity, surface charge and ion-binding capacity. By affecting any of these parameters, ciliary lipids may affect signal transduction by ciliary proteins, and perhaps especially ciliary membrane-associated proteins.

The ciliary membrane consists of a fraction of the cellular membrane, less than 0.01% of the total (Mukhopadhyay et al., 2017) and, to date, lipidomic characterizations of cilia have been restricted to those of organisms from which cilia can be collected in biochemical quantities (Lobasso et al., 2010; Raleigh et al., 2018). Previously, we fractionated membranes of sea urchin cilia from other cellular membranes and discovered that sea urchin cilia were enriched in several oxysterols, oxygenated derivatives of cholesterol (Raleigh et al., 2018).

Due to technical challenges in purifying mammalian primary ciliary membranes, we know less about which lipids compose vertebrate primary cilia than the cilia of protists and invertebrates. Techniques for determining the subcellular localization of lipids lag behind equivalent approaches for proteins. For example, proximity labeling approaches have greatly accelerated elucidation of the mammalian ciliary proteome (Mick et al., 2015). The ability to label lipids in specific subcellular domains does not currently exist, but its development would be a boon to comparing the lipid composition of many subcellular membranes, not just that of the ciliary membrane. Similarly, fluorescence imaging of lipids is hampered by the lack of molecular probes for most lipids (Balla and Várnai, 2002; Wills et al., 2018).

Because of the limitations to identifying ciliary lipids in vertebrate cells, we do not know whether the enrichment of sphingolipids and sterols extends to the many types of animal cilia. Indeed, staining of mammalian cilia for sterols has shown conflicting results about whether sterols are enriched (Nelson et al., 2008; Breslow et al., 2013; Kinnebrew et al., 2019; Miyamoto et al., 2020). Thus, sterol enrichment in cilia may be cell type-specific or be limited to a class of sterols detected by specific visualization methods.

However, like sea urchin, sea anemone, and protists, mammalian sperm can be fractionated into their heads, analogous to cell bodies, and tails, analogous to cilia (Toshimori et al., 1985; Connor et al., 1998; Mourvaki et al., 2010). Sterol levels in the sperm heads and tails differ, suggesting that, as in protists, lipids may be differentially distributed between the cilium and other subcellular compartments in animal cells.

CILIARY PHOSPHOINOSITIDES REGULATE GPCR DELIVERY AND HH SIGNALING

Recent reviews have described how lipids contribute to ciliary structure (Garcia et al., 2018; Nechipurenko, 2020). In this section, we focus specifically on how ciliary lipids participate in the transduction of HH signals, the best understood of the intercellular cues communicated via cilia. The best understood of the lipids participating in ciliary signaling are the phosphoinositides.

Phosphoinositides are phosphorylated lipids that confer molecular identity to cellular membranes (di Paolo and de Camilli, 2006; Shewan et al., 2011). Reversible phosphorylation of phosphatidylinositol can give rise to seven distinct phosphoinositides which exhibit distinct subcellular distributions (Schink et al., 2016). For instance, the Golgi membrane is enriched in PI(4)P, whereas the nuclear envelope is enriched in PI(5)P (Shewan et al., 2011). Physical separation of these membranes helps partition these distinct phosphoinositides.

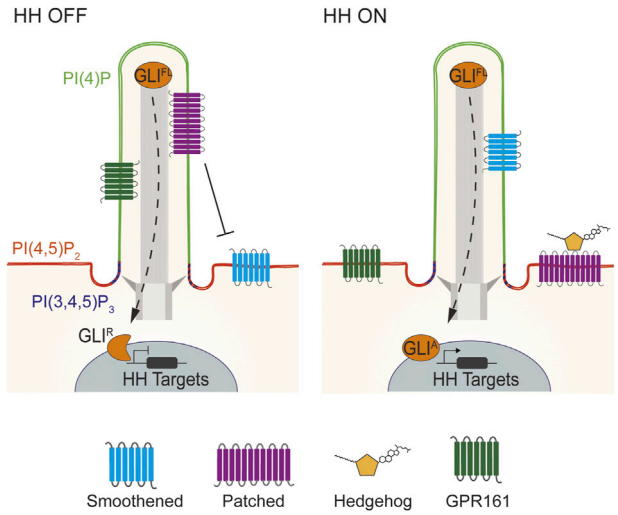

FIGURE 2 | Lipid domains within vertebrate cilia. Vertebrate HH signaling depends on the tightly coordinated trafficking of pathway components into and out of cilia. In the absence of HH signals, PTCH1 localizes to cilia and represses SMO. Consequently, GLI transcription factors are proteolytically processed to their repressor state to inhibit HH target genes. HH binding to PTCH1 leads to SMO accumulation in cilia, GPR161 exit, and GLI activator formation to induce HH target gene expression (Bangs and Anderson, 2017; Kong et al., 2019). Specialized membrane domains within mouse and human cilia allow for HH signal transduction. Mouse and human cilia are enriched in the phosphoinositide PI(4)P, whereas PI(4,5)P_2 is enriched outside of the cilium. Defects in the distribution of these two lipids causes mislocalization of HH pathway components such as GPR161 to cause birth defects (Chávez et al., 2015; Garcia-Gonzalo et al., 2015). In contrast to PI(4)P, PI(4,5)P_2 and PI(3,4,5)P_3 are enriched at the transition zone at the ciliary base (Dyson et al., 2017; Conduit and Vanhaesebroeck, 2020).

Thus, it is surprising that the phosphoinositide compositions of the ciliary and plasma membranes are distinct despite being contiguous, with the ciliary membrane being relatively enriched in PI(4)P and the plasma membrane relatively enriched in PI(4,5)P_2 (Conduit and Vanhaesebroeck, 2020; Conduit et al., 2021). An additional domain of PI(3,4,5)P_3 localizes near the ciliary base (**Figure 2**) (Dyson et al., 2017).

How might this phosphoinositide boundary be maintained? One strategy for maintaining distinct lipid compositions within a contiguous membrane is through control of the localization of lipid biosynthetic enzymes. In mammals, three phosphoinositide 5-phosphatases (INPP5E, INPP5B and OCRL) convert PI(4,5)P_2 into PI(4)P and localize to primary cilium (Bielas et al., 2009; Jacoby et al., 2009; Luo et al., 2012, 2013). Though these proteins may share overlapping functions, INPP5E is required to generate PI(4)P in the primary cilia of many cells (Chávez et al., 2015; Garcia-Gonzalo et al., 2015).

Maintenance of elevated PI(4)P and depleted PI(4,5)P_2 within the ciliary membrane is critical for HH signal transduction (Chávez et al., 2015; Garcia-Gonzalo et al., 2015; Dyson et al., 2017). Loss of INPP5E reduces ciliary PI(4)P and increases ciliary PI(4,5)P_2. The Tubby-family protein TULP3 binds PI(4,5)P_2 to control the delivery of a negative regulator of HH signaling, GPR161, to cilia. In the absence of INPP5E and ciliary PI(4)P, TULP3 and GPR161 mis-accumulate in cilia (Chávez et al., 2015; Garcia-Gonzalo et al., 2015). GPR161 activates protein kinase A (PKA), a negative regulator of GLI activity via direct binding to the regulatory subunit of PKA (PKA-R), and constitutive coupling to the G-protein Gαs, to generate cAMP, the principal PKA activator (Mukhopadhyay et al., 2010, 2013; Bachmann et al., 2016). We recently identified a pool of ciliary PKA (Truong et al., 2021). Thus, mis-activation of ciliary PKA to tonically inhibit GLI activity is likely to be how loss of INPP5E or ciliary PI(4)P suppresses HH signaling. Future work may elucidate how TULP3, and its paralogs including the obesity-associated protein TUBBY, read ciliary phosphoinositide composition to limit the ciliary localization of GPR161 and perhaps other GPCRs.

Aside from affecting GPCR localization, ciliary phosphoinositides may also directly affect GPCR function. Phosphoinositides can stabilize GPCR active states or enhance specific G-protein coupling (Yen et al., 2018). Differences in phosphoinositide composition between the ciliary and plasma membranes may allow cells to control GPCR output with spatial precision. For example, perhaps ciliary GPCRs, such as GPR161, may be tuned to be active specifically in domains rich in PI(4)P. And perhaps other GPCRs, such as FFAR4, which may operate at the ciliary membrane in preadipocytes and at the plasma membrane in adipocytes, may couple differently to G-proteins in these two different domains to allow for different outputs at different stages of differentiation (Hilgendorf et al., 2019).

CILIARY STEROLS ACTIVATE SMOOTHENED

Sterol lipids are a diverse class of lipids synthesized by the mevalonate pathway. Both cholesterol, the predominant sterol in vertebrate cells, and select oxysterols can bind to SMO to activate the HH pathway (Cooper et al., 2003; Dwyer et al., 2007; Myers et al., 2017). As SMO localization to primary cilia is required for activation of the HH pathway, the sterol composition of the ciliary membrane may contribute to SMO function. A recent study used a loss-of-function CRISPR-based approach to identify sterol biosynthetic genes that influence the strength of HH signaling (Kinnebrew et al., 2019). Liquid chromatography-tandem mass spectrometry of biochemically isolated sea urchin and porcine renal cells (LLC-PK1) helped to identify SMO-activating oxysterols enriched in cilia (Raleigh et al., 2018).

Unlike the case with phosphoinositides, there is not clear evidence of enriched localization of sterol or oxysterol catabolic enzymes at the cilium itself. However, a recent study identified sterol biosynthetic enzymes that localize at the ciliary base, including DHCR7 (Findakly et al., 2021). DHCR7 is mutated in Smith-Lemli-Opitz syndrome, an inherited disease characterized by holoprosencephaly. The holoprosencephaly is thought to be secondary to reduced HH signaling caused by the accumulation of SMO-inhibiting sterols (Fitzky et al., 1998; Wassif et al., 1998; Matsumoto et al., 2005; Nowaczyk and Irons, 2012; Sever et al., 2016). DHCR7 catalyzes the terminal

step in cholesterol and 24,25-epoxycholesterol synthesis. As an integral membrane protein, DHCR7 near the ciliary base may be in the ciliary pocket membrane, a membrane invagination that surrounds the cilium (Findakly et al., 2021). DHCR7 relocalizes away from the ciliary base upon HH pathway activation, suggesting that control of the subcellular localization of sterol biosynthetic machinery may modulate ciliary lipid composition to tune HH signaling. However, understanding how sterol content in cilia is controlled remains a major challenge, particularly as existing sterol biosensors are less specific than biosensors for other lipids such as phosphoinositides (Maekawa and Fairn, 2014; Wills et al., 2018).

HEDGEHOG LIGANDS ARE BOTH CHOLESTERYLATED AND PALMITOYLATED

Not only are lipids critical for creating specialized sub-cellular compartments that facilitate signaling, but lipids participate with certain core components of the HH pathway in ways critical for signaling. For example, HH proteins are covalently linked to palmitoyl and cholesterol (Porter et al., 1996a; Porter et al., 1996b; Pepinsky et al., 1998). Initially, HH is synthesized as a 45 kDa precursor comprised of a signal peptide, an N-terminal signaling domain (HhN) and a C-terminal intein (HhC) (Lee et al., 1994). Concurrent with synthesis, the signal peptide is cleaved, revealing a highly conserved N-terminal cysteine residue that is palmitoylated by Hedgehog acetyltransferase (called HHAT or SKI) (Pepinsky et al., 1998; Amanai and Jiang, 2001; Chamoun et al., 2001; Micchelli et al., 2002; Buglino and Resh, 2008). Additionally, the intein catalyzes HH cleavage and links cholesterol with the newly exposed C-terminus of HhN, thereby creating a fully processed, ~19 kDa protein that is dually lipidated (Porter et al., 1996a; Porter et al., 1996b; Pepinsky et al., 1998). Perturbing HH lipidation has different effects in vertebrates and in *Drosophila*, which we discuss in two broad categories: signaling activity and signal distribution.

PALMITOYLATION IS IMPORTANT FOR HH SIGNALING STRENGTH

The signaling potency of *Drosophila* HH and vertebrate SHH are differentially dependent on palmitoylation. In mouse fibroblast cells, non-palmitoylated SHH can still signal, albeit at reduced strength (Pepinsky et al., 1998). Similarly, non-palmitoylated SHH exhibits attenuated signaling *in vivo*, but, when overexpressed in the mouse embryonic limb bud, can, like overexpressed wild-type SHH, induce HH target genes and polydactyly (Lee et al., 2001; Chen et al., 2004).

In contrast to the mouse, un-palmitoylated Hh in *Drosophila* interferes with the signaling activity of wild-type Hh when globally overexpressed (Lee et al., 2001). Interestingly, this lack of activity seems to be specific to the ligand, and not to the system, since un-palmitoylated mouse SHH retains some signaling ability when ectopically expressed in the *Drosophila* wing disc (Chamoun et al., 2001). Un-palmitoylated HH can still partially rescue HH loss-of-function in the embryo (Gallet et al., 2003) and can induce HH signaling in the *Drosophila* wing disc (Callejo et al., 2006). Despite some species-specific dependence on palmitoylation, the palmitoyl moiety on Hedgehog proteins is critical for full signaling activity.

Cryo-EM structures of PTCH1 binding SHH reveal that SHH can bind in multiple conformations. In one conformation, the palmitoyl group makes extensive interactions in an extracellular cleft of PTCH1 composed of its two major extracellular loops, providing structural insight into one way that SHH blocks PTCH1 to activate the pathway (Qi et al., 2018a; Qi et al., 2018b; Qian et al., 2019).

HH CHOLESTERYLATION PROMOTES LONG-DISTANCE SIGNALING

In addition to binding PTCH1 to activate the downstream pathway, the developmental functions of HH ligands in tissue patterning depend on its distribution. In the neural tube, SHH forms a gradient, highest ventrally at its sites of production, the notochord and floor plate, and decreases dorsally. In the limb bud, SHH produced posteriorly in the zone of polarizing activity decreases in concentration anteriorly. Palmitoylation of vertebrate SHH is required for long-distance signaling as un-palmitolyated SHH is largely restricted to its sites of production (Lee et al., 2001; Chen et al., 2004). Importantly, both HH and SHH proteins that lack cholesterol are still competent to induce downstream transcriptional changes in receiving cells (Porter et al., 1996a; Lewis et al., 2001; Zeng et al., 2001; Li et al., 2006). Still, un-cholesterylated SHH cannot signal over long distances (Lewis et al., 2001). Thus, both lipid modifications are critical for vertebrate HH distribution, but cholesterylation may be more relevant to the range of signaling, rather than its signaling potency.

Drosophila demonstrate a cell-type specific requirement for lipidation, as un-cholesterylated Hh exhibits either restricted (Porter et al., 1996b; Burke et al., 1999; Dawber et al., 2005; Callejo et al., 2006; Gallet et al., 2006; Su et al., 2007) or expanded (Gallet et al., 2003, 2006; Panáková et al., 2005) spatial distribution in different tissues.

These differences in HH distribution in different organisms or tissues represents just one way in which HH signaling can be adapted. Another difference is the requirement for primary cilia in HH signal transduction. HH signal transduction in the *Drosophila* wing disc is independent of primary cilia. Indeed, wing disc cells lack cilia. In stark contrast, vertebrate HH signal transduction requires primary cilia (Huangfu and Anderson, 2005).

Additional vertebrate-specific requirements in HH signal transduction include the involvement of Scube-family proteins, vertebrate-specific extracellular proteins that facilitate HH release from producing cells. Scube proteins, though dispensable individually, are collectively required for HH signaling (Kawakami et al., 2005; Woods and Talbot, 2005; Hollway et al., 2006; Johnson et al., 2012). *In vitro*, SCUBE2 specifically

binds to and promotes the release of cholesteroylated SHH (Creanga et al., 2012; Tukachinsky et al., 2012; Wierbowski et al., 2020). Perhaps these species-specific differences in how HH signals are released from producing cells account for the different dependencies on lipidation for signaling by *Drosophila* HH and vertebrate SHH.

HH MAY COMMUNICATE OVER LONG DISTANCES VIA MULTIPLE MECHANISMS

How can HH act over multiple cell diameters as a morphogen once it is dually lipidated? As both lipid adducts on HH, cholesterol and palmitoyl, are poorly soluble in aqueous environments, HH would be expected to remain associated with membranes and not diffuse in the extracellular space. Conflicting results from studies done in *Drosophila*, zebrafish, and mouse are difficult to reconcile, raising the possibility that different organisms or different tissues distribute HH proteins in different ways. For example, there is evidence supporting the presence of HH in higher order assemblies that are less hydrophobic than monomeric lipidated HH, including as multimers, as constituents of liposomes, and as components of extracellular vesicles called exosomes.

One possibility is that HH multimerizes and internalizes its lipid moieties, exposing its hydrophilic proteinaceous face to the extracellular environment. *In vitro*, overexpressed HH will contribute to signaling-competent, high-molecular weight species in a way that depends on lipidation (Zeng et al., 2001; Chen et al., 2004; Gallet et al., 2006; Goetz et al., 2006). It remains unclear whether these high-molecular weight species exist *in vivo*.

It also is unclear whether proteins beyond HH contribute to these high-molecular weight complexes. Lipoprotein particles are extracellular macromolecular assemblies comprised of a core of esterified cholesterol moieties and triglycerides in association with apolipoproteins (Babin et al., 1999). HH can be released from *Drosophila* wing disc cells and human cultured cells as part of lipoprotein particles (Panáková et al., 2005; Palm et al., 2013). HH associated with liproprotein particles has low signaling activity (Palm et al., 2013), raising a question of whether this form of HH is critical to its function in developing tissues.

Additionally, HH may traffic on extracellular vesicles *in vitro*, in the *Drosophila* wing disc, and in developing mouse embryos (Tanaka et al., 2005; Matusek et al., 2014; Vyas et al., 2014). These extracellular vesicles may be formed via multivesicular body assembly or plasma membrane budding, mechanisms that are dependent on the endosomal sorting complex required for transport (ESCRT) (Matusek et al., 2014; Coulter et al., 2018). Whether these HH-containing extracellular vesicles have signaling capabilities and whether they can generate a morphogen gradient *in vivo* remain to be determined.

Some HH is not secreted but, rather, remains attached to the membrane and trafficked on long and thin cytonemes, specialized, actin-based cytoplasmic extensions as long as 200 μm (Kornberg, 2014). Cytonemes observed in the *Drosophila* wing disc correspond in length to the distribution of HH signaling and can also contain PTCH, raising the possibility that cytonemes can both send and receive signals (Bischoff et al., 2013; Gradilla et al., 2014). In the developing chick limb, cytonemes also contain HH, indicating that cytonemes may represent an evolutionarily conserved mechanism for distributing HH signals (Sanders et al., 2013). It will be of interest to specifically disrupt vertebrate cytonemes to assess how they shape HH signaling.

THE HH RECEPTOR, PTCH, TRANSPORTS STEROLS

Beyond HH itself, constituents of the HH signal transduction pathway are intimately associated with lipids. The HH receptor is a twelve-pass transmembrane protein called Patched (PTCH), of which most vertebrates have two homologs, PTCH1 and PTCH2 (Ingham and McMahon, 2001). PTCH proteins form a clade of the larger resistance-nodulation-division (RND) transporter-like family (Taipale et al., 2002). Bacterial RND proteins are exporters of diverse molecules that include hopanoids, sterol-like molecules (Tseng et al., 1999). In addition to PTCH, the RND family includes NPC1, a transporter which in animals conducts cholesterol across the lysosomal membrane (Kwon et al., 2009). Like NPC1, PTCH includes a sterol-sensing domain (SSD), implicated in the subcellular trafficking of sterols. Another similarity to NPC1 is that PTCH1 contains a hydrophobic channel that may contain sterols (Gong et al., 2018).

These structural similarities suggest that PTCH1 functions similarly to NPC1, validated by several cryo-EM-elucidated structures of the core of PTCH1 (Qi et al., 2018a; Qi et al., 2018b; Gong et al., 2018; Qi et al., 2019; Qian et al., 2019; Rudolf et al., 2019). Indeed, PTCH1 can efflux a fluorescent form of cholesterol and SHH inhibition of PTCH1 increases intracellular cholesterol concentration (Bidet et al., 2011). Structural analysis reveals that PTCH1 interacts with sterols at ten or more sites and can partially lift sterols out of the membrane bilayer (Qi et al., 2019). Although the functional importance of the partial removal of a sterol from the membrane is unclear, it may represent an intermediate step in sterol transport. Indeed, PTCH1 can transport lipid sterols away from the inner leaflet of the membrane (Zhang et al., 2018; Qi et al., 2019) and it is likely that the binding of PTCH1 to HH blocks PTCH1 to allow buildup of a SMO-activating sterol, perhaps specifically in the ciliary membrane, thereby activating the downstream signal transduction pathway.

Numerous PTCH1 mutations associated with the human birth defect holoprosencephaly increase its ability to inhibit SMO (Petrov et al., 2021). Loss-of-function mutations in PTCH1 cause misactivation of SMO and some forms of cancer (Gailani et al., 1996; Hahn et al., 1996; Johnson et al., 1996). Whether either set of missense mutations alter sterol transport will be interesting to assess.

Other hints about PTCH function can be gleaned from evolutionary perspectives. Some bilateria, notably *Caenorhabditis elegans*, have lost the HH pathway but retained PTCH homologs. One of these, PTR-18 clears a secreted protein, GRL-7, distantly related to HH (Chiyoda et al., 2021), suggesting

that PTCH can be repurposed to function independently of HH pathway regulation. Another *C. elegans* PTCH homolog, PTC-3, prevents intracellular cholesterol accumulation (Cadena del Castillo et al., 2021), further supporting the idea that PTCH family members are sterol transporters.

Interestingly, a paralog of PTCH cleverly called Dispatched1 (DISP1) functions not in HH reception but in transmitting HH from the cells in which it is produced (Burke et al., 1999). DISP1 forms a sodium channel and depends on the sodium gradient to release SHH from producing cells, raising the possibility that flux of sodium down its chemiosmotic gradient may power the extraction of cholesteroylated HH from the membrane (Petrov et al., 2020; Wang et al., 2021). Recent structures of DISP1 reveal that, like PTCH1, it partially displaces a sterol from the membrane bilayer (Wang et al., 2021). This lifted sterol may represent an ability of DISP1 to pry the cholesterol adduct of HH out of the plasma membrane, potentially a step in its transfer of HH to SCUBE2.

Many of the residues involved in coordinating sodium are also present in PTCH1, consistent with evidence that a sodium or potassium gradient is critical to the ability of PTCH1 to suppress the signaling activity of SMO (Myers et al., 2017; Petrov et al., 2020). It will be interesting to determine how PTCH1 uses a monovalent cation gradient. Perhaps cation flux through PTCH1 powers the removal of SMO-activating sterols from the ciliary membrane in a way that is analogous to RND-mediated export of hopanoids from the inner membrane of bacteria.

In addition to PTCH, HH is bound by additional proteins not essential for all HH communication, including HHIP, CDON, BOC, GAS1 and LDL receptor-related protein 2 (LRP2) (Chuang and McMahon, 1999; Stebel et al., 2000; Yao et al., 2006; Zhang et al., 2006; Christ et al., 2012). These auxiliary HH-binding proteins operate differently from each other: HHIP negatively regulates HH signaling while the others potentiate HH signaling (except for in the retina, where LRP2 inhibits HH signaling) (Christ et al., 2015).

As its name implies, LRP2 is a member of the family of low-density lipoprotein (LDL) receptors. LRP2 is required, like SHH, for forebrain development in mice (Willnow et al., 1996). Inherited mutations of *LRP2* in humans cause Donnai-Barrow syndrome, which includes craniofacial defects that may be related to altered HH signaling (Kantarci et al., 2007, 2008).

Some other LRP family members also function in developmental pathways. For example, LRP5 and LRP6 are part of the WNT receptor complex (Pinson et al., 2000). WNT ligands, like HH, are palmitoylated (Willert et al., 2003). The best studied member of the family, LDLR, binds and endocytoses LDL, bringing cholesterol into the cell. In addition to HH, LRP2 binds to a variety of ligands, including proteins that carry steroid-like molecules (Christensen et al., 1999; Nykjaer et al., 1999; Hammes et al., 2005).

Where do ciliary lipids come from? In animals, cholesterol is generated within the cytosol and endoplasmic reticulum (ER) or delivered via LDLs. Upon uptake, LDL is endocytosed and fused with lysosomes to release cholesterol for delivery to the plasma membrane (Brown and Goldstein, 1986). A key regulator of plasma membrane cholesterol content is NPC1, mutated in Neiman-Pick disease. Mice lacking NPC1 show decreased ciliogenesis and shortened cilia, with decreased HH signaling in the cerebellum, raising the possibility that NPC1 helps deliver cholesterol to the ciliary membrane (Canterini et al., 2017). However, NPC1 is not generally required for HH signaling, indicating that either there are NPC1-independent mechanisms of delivering cholesterol to the ciliary membrane or that NPC1-dependent ciliary cholesterol is not essential for HH pathway activation.

The endocytosis of a variety of lipid-associated proteins via LRP family members raises the possibility that internalization of extracellular lipids was the original role for these proteins. Although speculative, it is possible to imagine that extracytosolic lipid-binding proteins, functionally akin to the evolutionarily ancient tubular lipid-binding proteins (TULIPs) or the more recently evolved cholesterol carrier NPC2, might have facilitated lipid uptake (Wong and Levine, 2017). Perhaps upon acquisition of multicellularity and increased needs for cell-cell communication, these extracellular lipid-binding proteins became lipoprotein receptors and acquired new roles in information transmission. The genomes of the simple animals, such as *Trichoplax*, sea anemones and sponges, encode members of the LRP family member (e.g., TRIADDRAFT_27379, TRIADDRAFT_19424, A0A1X7TVZ2), suggesting that LRP proteins arose before porifera and placozoa split from each other early in the evolution of multicellular animals. Thus, it is possible that evolution acted on a system for lipid nutrient uptake, converting it into systems for cell-cell communication such as WNT and HH signaling.

Like LRP proteins, a canonical HH pathway is present in many basal animals, including sponges and sea anemones, but is absent from choanoflagellates and other single-celled eukaryotes (**Figure 1**). Despite the absence of the complete HH pathway in protists, PTCH homologs are present in some protist genomes, raising the intriguing possibility that PTCH is the most evolutionarily ancient member of the pathway and was subsequently co-opted for HH signal transduction. For example, *Chlamydomonas* possesses two PTCH orthologs (Cre02.g093500 and Cre12.g496350) which, unfortunately, have not been studied.

The main sterol in *Chlamydomonas* membranes is not cholesterol, but ergosterol (Gealt et al., 1981). It will be interesting to discover whether protist PTCH family members share the interaction with sterols with their metazoan cousins. As yeast NPC1 transports ergosterol and animal NPC1 transports cholesterol, it is possible that PTCH has similarly evolved to transport different sterols in different organisms. Interestingly, one *Chlamydomonas* flagellar lipid, an ergosterol endoperoxide, can inhibit mammalian HH signaling (Sever et al., 2016), raising the possibility that protist PTCH homologs could act on sterols with sufficient similarity to animal sterols that they can interact with the mammalian HH signal transduction pathway. Perhaps elucidating the functions of protist PTCH homologs will provide insights into the types of sterols transported by these elusive channels.

CHOLESTEROL AND OXYSTEROLS CAN ACTIVATE SMOOTHENED

PTCH suppresses the function of SMO, the central positive activator of the downstream HH signal transduction pathway.

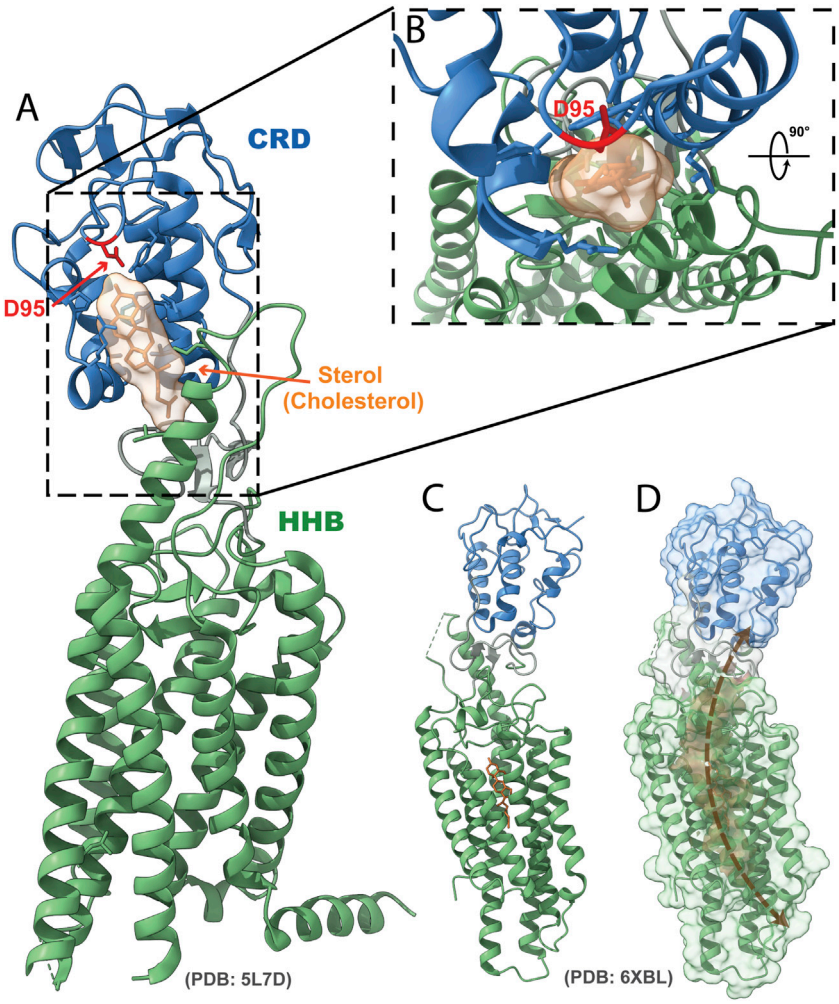

FIGURE 3 | Structure of SMO bound to sterols. **(A)** Ribbon representation of the crystal structure of human SMO bound to a sterol in the CRD, with cholesterol depicted (PDB: 5L7D). Blue, CRD; green, HHB; orange, sterol; red, residue D95. **(B)** Inset depicts a top-down view of the CRD binding pocket with a sterol bound. **(C)** Structure of human SMO with one of the putative sterol-binding sites in the HHB depicted (PDB: 6XLB). **(D)** Surface rendering of the potential sterol channel in SMO.

SMO is comprised of an N-terminal, extracellular cysteine-rich domain (CRD), an extracellular linker domain, a transmembrane heptahelical bundle (HHB), and a C-terminal cytosolic tail.

How might PTCH inhibit SMO activity? Previous hypotheses posited that PTCH directly binds to and sequesters SMO in a way that is relieved upon HH binding to PTCH (Stone et al., 1996; Murone et al., 1999). However, PTCH and SMO do not interact tightly and have distinct subcellular distributions, even in the primary cilium (Denef et al., 2000; Corbit et al., 2005; Rohatgi et al., 2007). Moreover, PTCH can inhibit SMO sub-stoichiometrically, with half-maximal pathway activity observed only when SMO was in 50-fold molar excess of PTCH (Taipale et al., 2002). These data, combined with the ability of PTCH to transport sterols (Zhang et al., 2018; Qi et al., 2019), suggests that PTCH may export a SMO-activating sterol.

Like PTCH, SMO binds sterols at several sites (**Figures 3A,C**) (Myers et al., 2013; Rana et al., 2013; Byrne et al., 2016; Huang et al., 2016, 2018; Luchetti et al., 2016; Raleigh et al., 2018; Deshpande et al., 2019). SMO mutations that alter individual sterol sites, either within the CRD or HHB, compromise HH signal transduction (Myers et al., 2013; Nachtergaele et al., 2013; Raleigh et al., 2018), suggesting that sterol binding is important for signal transduction.

How sterols activate SMO binding remains unclear. Although unprecedented for a GPCR-like protein, one possibility is that these binding sites form a continuous intramolecular channel through SMO capable of sterol transport (**Figures 3C,D**) (Huang et al., 2018; Qi et al., 2020). Mutations in the HHB that are likely to prevent sterol movement within SMO constitutively activate signaling (Qi et al., 2020). Perhaps these mutations block sterol transit through SMO and increased sterol occupancy within SMO is sufficient to activate SMO.

In addition to interacting with sterols, SMO can be covalently modified by cholesterol at the CRD (Xiao et al., 2017). This cholesterylation occurs in human SMO at the D95 residue (mouse SMO D99) within the CRD sterol binding site (Byrne

et al., 2016) (**Figures 3A,B**). Mutation of this aspartic acid to hinder cholesterol modification of SMO compromises ciliary localization and signaling (Xiao et al., 2017; Hu et al., 2022). Thus, it is likely that covalent binding of SMO to cholesterol stabilizes its active state. It will be interesting to establish whether both non-covalent and covalent interaction with cholesterol are sufficient to promote pathway activity *in vivo*.

The cholesterylation of SMO is inhibited by PTCH1 and promoted by HH ligand (Xiao et al., 2017). Understanding where within the cell SMO is cholesterylated (e.g., before or after ciliary localization) will help reveal how sterols affect HH signaling. As SMO lacking the CRD domain is still able to weakly activate the downstream pathway (Raleigh et al., 2018), sterol interaction with the CRD is likely to be a modulatory effect on SMO activity.

Both oxysterols and cholesterol can bind SMO. However, it is still an open question which sterols activate SMO *in vivo*. Indeed, the SMO-activating sterol may be cholesterol, oxysterol, or some combination thereof. Given that cholesterol is a highly abundant lipid in the plasma membranes of animal cells and quickly transits between the inner and outer leaflets, it is unclear how PTCH1 could inhibit cholesterol accumulation specifically in the outer leaflet to prevent SMO misactivation. One possibility is that much of the membrane-associated cholesterol is sequestered as a form that cannot regulate SMO (Kinnebrew et al., 2019; Radhakrishnan et al., 2020). Thus, the pool of cholesterol relevant to SMO regulation ("accessible" cholesterol) may be smaller than the total cholesterol pool.

Certain oxysterols [e.g., 7β,27-DHC, 24k-C, and 24(S),25-EC] are enriched in the primary cilium, can bind to SMO, and can promote the accumulation of SMO in cilia, and thus are candidate regulators of SMO activation (Raleigh et al., 2018). As SMO possesses multiple sterol binding sites (Myers et al., 2013; Raleigh et al., 2018; Qi et al., 2020), multiple sterols may be relevant even to a single molecule of SMO. Indeed, it could even be possible that the same sterol could antagonize SMO function when binding at or near the orthosteric site within the heptahelical core and agonize SMO when binding the extracellular CRD.

CONCLUSION

In this review, we have summarized the intimate connection between HH signaling and lipids. Lipids participate in the HH-mediated orchestration of developmental and homeostatic processes at multiple levels, including as constituents of cellular membranes, as ligands or substrates for key pathway components, and as covalent modifiers of HH and SMO.

Cilia are evolutionarily ancient organelles which possess a distinct ciliary lipid composition in organisms as diverse as *Chlamydomonas*, *Tetrahymena* and *Paramecia*. In vertebrates, primary cilia also have a unique lipid composition, including enrichment in PI(4)P. Vertebrate HH signal transduction depends on primary cilia, and on the lipids of the primary cilium, bringing a subcellular focus to many steps of HH signal transduction.

Despite remarkable advances, our understanding of the role and regulation of lipids trails our understanding of proteins. The development of new tools to detect and perturb specific lipids will diminish this gap. For example, specific and sensitive lipid biosensors will permit visualization of the spatial distribution of the ciliary lipid composition. To help unravel how lipids function in HH signaling, it will be particularly helpful to develop sterol biosensors, refine mass spectrometry-based lipidomic approaches, and create optogenetic or chemogenetic approaches to specifically deplete lipids in subcellular domains such as the primary cilium. Especially in the emerging era of superresolution microscopy, identification of lipid domains and how they are dynamically regulated may be in the offing. As we have some understanding of how HH signals remodel ciliary protein composition, it will be particularly interesting to assess whether HH signals also dynamically remodel the ciliary lipid composition to activate signaling.

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