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Sterol regulation of developmental and oncogenic Hedgehog signaling

Vikas Daggubati[1,2], **David R. Raleigh**[1], **Navdar Sever**[3,*]

[1]Departments of Radiation Oncology and Neurological Surgery, and Biomedical Sciences Graduate Program, University of California, San Francisco, CA, USA

[2]Medical Scientist Training Program, University of California, San Francisco, CA, USA

[3]Department of Cell Biology, Harvard Medical School, Boston, MA, USA

Abstract

The Hedgehog (Hh) family of lipid-modified signaling proteins directs embryonic tissue patterning and postembryonic tissue homeostasis, and dysregulated Hh signaling drives familial and sporadic cancers. Hh ligands bind to and inhibit the tumor suppressor Patched and allow the oncoprotein Smoothened (SMO) to accumulate in cilia, which in turn activates the GLI family of transcription factors. Recent work has demonstrated that endogenous cholesterol and oxidized cholesterol derivatives (oxysterols) bind and modulate SMO activity. Here we discuss the myriad sterols that activate or inhibit the Hh pathway, with emphasis on endogenous 24(S),25-epoxycholesterol and 3β,5α-dihydroxycholest-7-en-6-one, and propose models of sterol regulation of SMO. Synthetic inhibitors of SMO have long been the focus of drug development efforts. Here, we discuss the possible utility of steroidal SMO ligands or inhibitors of enzymes involved in sterol metabolism as cancer therapeutics.

Keywords

medulloblastoma; basal cell carcinoma; Sonic hedgehog; sterol-sensing domain; oncogene

1. Introduction

Discovered in *Drosophila melanogaster*, the Hedgehog (Hh) pathway plays an essential and conserved role in tissue development and homeostasis across species [1]. Genetic and biochemical studies, first in *Drosophila* and later in vertebrates, revealed that Hh proteins (Sonic hedgehog, Desert hedgehog and Indian hedgehog in mammals) are secreted with two covalently attached lipid moieties: a palmitoyl moiety at the N terminus and a

*Corresponding author: Navdar Sever, Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, LHRRB 405, Boston, MA 02115, USA, Navdar_Sever@hms.harvard.edu, Telephone: (617) 432-1612.

Conflict of Interest

The authors declare that no conflict of interest exists.

cholesterol molecule at the C terminus. Secreted Hh proteins act on distant cells by diffusing through the extracellular environment with the aid of proteins that shield the hydrophobic lipids [2]. The Patched receptor (Ptc in flies and PTCH1 or PTCH2 in vertebrates) on recipient cells interacts with Hh proteins, which relieves suppression of Smoothened (SMO) and activates the glioma-associated oncogene family of transcription factors (Ci in flies and GLI in vertebrates; Fig. 1). Although core Hh pathway components are conserved across species, mechanisms of Hh signal transduction have diverged between flies and mammals [3]. First, canonical mammalian Hh signaling requires a microtubule-based organelle called the primary cilium, and many Hh pathway components, such as PTCH, SMO, and GLI, dynamically localize to cilia depending on pathway activation [4]. Second, SMO activation in mammals requires cellular sterols, which is demonstrated by loss of Hh pathway activation after depletion of sterols from the plasma membrane [5]. Although recent structural studies using X-ray crystallography and cryo-electron microscopy (cryo-EM) have provided insights into mechanisms of PTCH regulation of SMO in mammals (reviewed in [2]), the precise role of cholesterol and its metabolites remains poorly understood and controversial due to the lack of tools to specifically manipulate endogenous sterols. However, misactivation of Hh signaling causes both familial and sporadic cancers [6]. Thus, understanding the role of lipid metabolites and the regulation of these compounds in the Hh pathway is vital for improving our knowledge of basic mechanisms of Hh signal transduction, and represents an unexplored avenue for novel therapeutics.

2. (A)steroid impact on SMO

SMO is a Frizzled (FZD) family G protein-coupled receptor (GPCR) and contains a seven-transmembrane (7TM) domain and an extracellular cysteine-rich domain (CRD; Fig. 1). In all other FZD family receptors, the CRD binds to the Wnt family of lipid-modified signaling proteins, where a palmitoleate moiety on Wnt is directly involved in CRD binding [7]. Compared to all other known GPCRs, the 7TM domains of FZDs have a narrow pocket that is not amenable to binding small molecules and is considered to be undruggable [8,9]. Hh ligands are also covalently modified by lipids, but Hh proteins bind to PTCH instead of SMO and the role of SMO CRD is controversial. These observations raise a fundamental question: how does PTCH regulate SMO activity? PTCH is a 12TM transporter-like protein (see Section 4 below), and one alluring hypothesis is that PTCH regulates the abundance of a small molecule regulating SMO activity. In support of that hypothesis, SMO has at least 4 different small molecule binding sites, one in CRD and three within the 7TM domain, that have been structurally and biochemically shown to bind cholesterol, several oxysterols, and numerous synthetic agonists and antagonists of Hh pathway [2,10–13] (Fig. 1). The first small molecules identified to bind SMO were the steroidal plant compounds cyclopamine and jervine [14]. Fluorescent cyclopamine derivatives were shown to bind the 7TM domain, and used to find other small molecules that would compete for binding at the same site [15]. This approach led to the discovery of several synthetic small molecule antagonists, including vismodegib, sonidegib and the cyclopamine derivative saridegib, that are either approved as cancer therapeutics or are currently under investigation in clinical trials [16]. More recently, the SMO CRD and 7TM domains were shown to bind a partially overlapping set of sterols (see Fig. 3). Currently, 24(S),25-epoxycholesterol holds the record for the number

of sites it has been shown to bind within SMO. Two molecules of 24(S),25-epoxycholesterol are observed to simultaneously bind sites 2 and 3 in one cryo-EM structure [12], and both we [11] and Qi et al. [12] showed biochemically that it can also bind CRD (site 4, Fig. 1). Residue N521 of SMO interacts with the epoxy tail of 24(S),25-epoxycholesterol bound at site 2, and when this residue is mutated, SMO can no longer be activated by 24(S),25-epoxycholesterol. It remains to be determined if this mutant can be activated by other sterols or Shh. In sum, SMO is regulated by a divergent mechanism compared to other FZD GPCRs, and it remains to be understood how endogenous SMO ligands mediate PTCH regulation of SMO.

Enzymatic oxidation products of cholesterol are one class of compounds that are of particular interest in regulating SMO activity. Historically thought of as intermediate metabolites in bile acid or steroid hormone biosynthesis, oxysterols are now known to act as important intracellular and intercellular signaling molecules [17]. A precedent for a GPCR with oxysterols as *bona fide* ligands is EBI2 [18,19]. EBI2 does not have a large extracellular domain, and presumably, oxysterols bind to the orthosteric ligand binding pocket found within the 7TM domain of most GPCRs. The most potent EBI2 agonist is 7α,25-dihydroxycholesterol, with an affinity (K_d) of ~1 nM, which is close to the endogenous concentration of oxysterols. Depletion of endogenous 7α,25-dihydroxycholesterol due to genetic deficiencies in either oxysterol 7α-hydroxylase (*Cyp7b1*) or cholesterol 25-hydroxylase (*Ch25h*) demonstrates a physiological requirement of 7α,25-dihydroxycholesterol for activating EBI2 [20]. Thus, SMO activity too may be modulated by an endogenous sterol and PTCH may act to regulate the availability of this endogenous ligand. However, it is unclear whether the putative substrate of PTCH is an agonist (activator) or antagonist (inhibitor) of SMO (green or red arrows, respectively, Fig. 2). Regardless of the identity of the endogenous SMO ligand, one interpretation is that the four ligand binding sites of SMO constitute a continuous tunnel, and the CRD facilitates access or removal of sterols that bind to the SMO 7TM domain. The role of the CRD in responding to Hh stimulation, however, would have to be auxiliary rather than obligatory as PTCH can still inhibit the activity of SMO constructs lacking the CRD [10,21].

3. Good sterol, bad sterol

There are three plausible models for how PTCH may regulate SMO: (1) PTCH inhibits SMO by reducing the accessibility of (ciliary) cholesterol, (2) PTCH removes an oxysterol agonist from SMO, and (3) SMO requires cholesterol but PTCH inhibits SMO via a sterol antagonist (Table 1). Model 1 is the prevailing model and posits that cholesterol is necessary and sufficient for SMO activation, and PTCH reduces the accessibility of cholesterol to SMO [22] (green arrows, Fig. 2). Cholesterol is observed around the 7TM bundle of many GPCR structures and, in some cases, modulates their activity by increasing binding affinities for orthosteric ligands [23,24]. In the case of oxytocin receptor, this was shown using methyl-β-cyclodextrin (MCD), a macrocyclic compound that selectively extracts sterols from the plasma membrane [24]. MCD was similarly used to demonstrate a sterol requirement for SMO function [5]. Moreover, cholesterol is present in large concentrations to act as a signaling molecule as it constitutes close to 50% of lipids in the plasma membrane [25], and two studies using toxin-based cholesterol sensors, such as

perfringolysin O (PFO), suggested PTCH affects cholesterol accessibility to SMO (Model 1). One of these studies proposed that PTCH flips cholesterol from the inner membrane to the outer membrane when expressed heterologously in HEK293 cells [26]. Another study using a mutant version of PFO and other sensors suggested that physiological levels of PTCH reduces cholesterol accessibility in only the ciliary membrane [27]. However, the general conclusions of the former study has been challenged for the quantitative accuracy of PFO [28]. Moreover, there are other putative cholesterol transporters, and likely ciliary receptors that are cholesterol-sensitive, raising the question of how PTCH could inhibit SMO *specifically* with such a mechanism. It is unlikely that PTCH removes ligands directly from SMO for two reasons. First, PTCH and SMO do not physically interact [29], nor colocalize, as they dynamically segregate in distinct domains within the ciliary membrane [30]. Second, one PTCH molecule is estimated to catalytically inhibit ~50 molecules of SMO [29], which also raises the question of the ultimate fate of the ligands that would be removed from SMO. In particular, cholesterol is insoluble and could not leave the membrane freely, and no protein has been implicated to cooperate with PTCH by serving as a sink for cholesterol. Thus, although the prevailing model suggests PTCH regulates SMO by regulating cholesterol accessibility, there remain fundamental open questions that need to be answered to support this model.

First, the reagents used to study the cholesterol's role in Hh signaling are imperfect. Both MCD and PFO are promiscuous tools, as the former is known to extract sterols other than cholesterol and the latter is known to bind to diverse sterols, including desmosterol and sitosterol [31–33]. In addition to 24(S),25-epoxycholesterol mentioned above, the search for putative endogenous oxysterols that modulate SMO activity led to the discovery of isomers of EBI2 ligands, 7-keto-25-hydroxycholesterol, 7-keto-27-hydroxycholesterol, 7β,25-dihydroxycholesterol and 7β,27-dihydroxycholesterol, as CRD agonists [10,11,34] (Figs. 3 and 4). Therefore, a second possibility is that the physiologic substrate of PTCH is not cholesterol, but another sterol that is a specific regulator of SMO (Model 2). Indeed, sterols such as 24-ketocholesterol and 24(S),25-epoxycholesterol were both detected by targeted mass spectrometry of lipids extracted from purified PTCH1 protein [35], and found to be enriched in cilia purified from sea urchins [11]. There are also precedents for a distinct sterol composition in eukaryotic cilia or flagella compared to the rest of the plasma membrane. For instance, in spermatozoa of some species, desmosterol is found in large amounts (in some cases exceeding cholesterol) and is exclusively confined to the flagella, which are specialized cilia [36–40]. However, the effective concentration of oxysterols required to activate SMO is orders of magnitude higher than their endogenous levels. Second, unlike mice lacking enzymes that catalyze distal steps of the cholesterol biosynthesis pathway, triple-knockout mice deficient in cholesterol 24-hydroxylase (*Cyp46a1*), *Ch25h* and sterol 27-hydroxylase (*Cyp27a1*) do not show developmental defects associated with reduced Hh signaling [41]. These mice have dramatically reduced levels of side-chain oxysterols, but some oxysterols may still be synthesized by redundant enzymes or non-enzymatic reactions.

24(S),25-epoxycholesterol is exceptional with respect to both arguments against Model 2. First, the levels of endogenous 24(S),25-epoxycholesterol range from 0.1-1 % of cholesterol, which corresponds in liver to 10-30 μM, well within its effective concentration

for SMO activation *in vitro* [42,43]. Second, 24(S),25-epoxycholesterol can be synthesized either from desmosterol by CYP46A1, or by a shunt pathway that does not require any additional enzyme besides those required for cholesterol synthesis [43,44] (Fig. 4). In fact, compared to this shunt pathway, cholesterol synthesis requires an additional enzyme, 24-dehydrocholesterol reductase (DHCR24), that converts desmosterol to cholesterol. In a CRISPR screen, Kinnebrew et al. [27] used the finding that loss of *Dhcr24* blocks Hh signaling to argue that cholesterol rather than 24(S),25-epoxycholesterol regulates SMO. However, they were not able to rescue Hh signaling in *Dhcr24*$^{-/-}$ cells by exogenous cholesterol, in contrast to the successful rescue in mutant cells deficient in other enzymes such as 7-dehydrocholesterol reductase (DHCR7). The lack of response to Shh in *Dhcr24*$^{-/-}$ cells is surprising because desmosterol itself can support SMO activation [45], and clear Hh-related phenotypes are not observed in the human disease desmosterolosis caused by mutations in *DHCR24* [46]. This is in contrast to mutations in *DHCR7* that cause Smith-Lemli Opitz syndrome (SLOS) with a subset of the developmental abnormalities observed in mice and humans with Shh mutations, such as holoprosencephaly [47]. One possibility is that desmosterol has an adverse but indirect effect on Hh signaling, for instance by disrupting lipid rafts, which cannot be alleviated by excess cholesterol [48]. Thus, further work needs to be done to test the involvement of endogenous 24(S),25-epoxycholesterol in Hh signaling. One way to accomplish this would be by adding diepoxysqualene, the committed precursor of 24(S),25-epoxycholesterol, and/or manipulating lanosterol synthase activity. As lanosterol synthase has a higher affinity for diepoxysqualene than monoepoxysqualene, partial inhibition of this enzyme has been shown to divert the metabolic flux from cholesterol biosynthesis towards the shunt pathway [49–51].

A third possibility is that SMO activity requires cholesterol (or another oxysterol agonist) but PTCH inhibits SMO via a sterol antagonist (Model 3, Table 1). A sterol requirement for SMO function has been demonstrated using two experimental protocols: (i) by acute treatment with a high concentration of MCD followed by chronic treatment with 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors, where HMG CoA reductase catalyzes the rate-limiting step of cholesterol synthesis, or (ii) by chronic treatment with a lower concentration of MCD [5]. Using the first protocol, 100 μM cholesterol added in ethanol (after MCD treatment) had been shown previously to rescue the response to Shh, indicating that cholesterol has access to SMO under these conditions but was not sufficient to activate SMO in the absence of Shh [10]. MCD can also be used as a delivery vehicle by solubilizing cholesterol in the form of an inclusion complex. Using the second protocol, the ability of MCD-cholesterol (100-250 μM final cholesterol concentration) to activate Hh pathway in the absence of any other agonist has been used to propose that cholesterol is not only necessary but also sufficient to activate SMO [45,52]. This is a misinterpretation based on the assumption that MCD is an inert delivery vehicle. An inclusion complex of MCD with a sterol effectively exchanges the included sterol with cellular sterols. In other words, MCD-cholesterol not only increases the cholesterol content of the plasma membrane but also extracts other sterols. MCD is only needed to deliver cholesterol to intracellular locations such as the endoplasmic reticulum (ER). It is well established that cholesterol added from an ethanol stock can rescue the growth of cholesterol auxotrophic cell lines, indicating that it can at least be incorporated into the

plasma membrane where it can serve its essential functions [53]. The fact that cholesterol in MCD, but not in ethanol, activates Hh pathway in the absence of Shh suggests that MCD could extract both positive and negative sterols. Since positive sterols are required for SMO activity, the net effect of empty MCD treatment is inhibition. When an MCD-cholesterol complex is used, the negative sterols are still extracted but the excess cholesterol allows SMO to be fully active. These data give rise to the possibility that PTCH regulates SMO, at least in part, via a sterol antagonist (Model 3, Table 1). Further support for an endogenous SMO antagonist came from a study demonstrating non-cell-autonomous inhibition of Hh response by PTCH from neighboring cells [54]. PTCH-expressing cells displayed an elevated Hh pathway inhibitory activity when the *Dhcr7* gene was mutated, consistent with the idea that 7-dehydrocholesterol or a derivative inhibits SMO. As PTCH cannot deplete sterols from other cells, this suggests that PTCH exports a SMO antagonist. Further, we showed that the activity of the synthetic SMO agonist (SAG) (Fig. 1) is potentiated by MCD, but not β-cyclodextrin or hydroxypropyl-β-cyclodextrin, even though all three cyclodextrins can extract cholesterol and inhibit Shh stimulation [55]. The ability of different cyclodextrins to potentiate SAG correlates instead with their ability to extract 7-dehydrocholesterol.

Consistent with the idea that MCD extracts a SMO inhibitor, some oxysterols (class I), but not others (class II), are more efficacious in stimulating SMO activity when delivered as MCD complexes (Fig. 3). This is unlikely to be related to efficiency of delivery as most oxysterols are positional isomers with similar physicochemical properties, such as solubility that is orders of magnitude higher than cholesterol. Rather, the difference between the two classes of sterols seems to be their binding site on SMO. Class I sterols act on SMO 7TM domain (even though some of them can also bind CRD) and have no effect when added in ethanol but can fully activate Hh pathway when delivered as an MCD complex. In contrast, class II sterols, which act on SMO CRD, are not more potent when delivered in MCD *vs* in ethanol. Further, a synergistic effect is seen when a class I sterol and a class II sterol are added together from ethanol stocks at low micromolar concentrations, indicating class I sterols do not need MCD to access their binding site within SMO 7TM domain [10,56]. Rather, as in the case of MCD-cholesterol complex, MCD extraction of an endogenous antagonist permits class I sterols to activate SMO. On the other hand, CRD agonists (class II oxysterols) apparently can activate SMO despite the presence of the putative antagonist.

A search for putative endogenous antagonists led to the discovery of oxidized derivatives of 7-dehydrocholesterol such as 3β,5α-dihydroxycholest-7-en-6-one (DHCEO), which accumulates in SLOS [55,57] (Fig. 5). DHCEO is formed in a three-step process from 7-dehydrocholesterol that parallels a known biotransformation of cholesterol. Although the initial product 5,6-epoxycholesterol does not require enzyme activity, it is converted by cholesterol-5,6-epoxide hydrolase (ChEH) to cholestane-3β,5α,6β-triol, which is then transformed into 6-oxo-cholestan-3β,5α-diol by 11β-hydroxysteroid dehydrogenase type 2 (HSD11β2), the same enzyme that interconverts CRD agonists in addition to converting cortisol to cortisone [11,58] (Figs. 4 and 5). Interestingly, ChEH is a complex between DHCR7 and emopamil-binding protein (EBP), which is another enzyme involved in cholesterol biosynthesis [59]. Therefore, in the absence of DHCR7, the analogous step in DHCEO biosynthesis must be carried out by one of the other known epoxide hydrolase

enzymes [60]. Although there is evidence for an enzymatic basis for the last step of DHCEO synthesis as well [61], it is not yet known if it is also carried out by HSD11β2. In various cultured cell and animal models, both cholesterol deficiency and 7-dehydrocholesterol accumulation have been shown to contribute to SLOS phenotype [62–65]. The current standard of care for SLOS is cholesterol supplementation, which also reduces accumulation of cholesterol precursors via feedback inhibition [66]. A combined supplementation of cholesterol with antioxidants that block the formation of abnormal oxysterols is currently under investigation in clinical trials [67]. Indeed, DHCEO accumulates to a concentration of ~3.5 μM in tissues of SLOS patients, but it is not clear whether the 100-fold lower concentration of DHCEO in normal tissues would be sufficient to regulate SMO activity [57].

Altogether, how SMO is regulated by sterols to activate the downstream Hh pathway remains unclear. Here we posit three models that all have varying levels of data that support or undermine their plausibility. However, these models are not mutually exclusive; i.e., an antagonist might displace positive sterols bound to SMO, or PTCH might remove an agonist from SMO in addition to providing the antagonist. As monitoring and manipulating endogenous lipids remain technically challenging, both technical innovations and novel insights are necessary to elucidate how sterols regulate SMO.

4. The origin story of a super family

The idea that PTCH acts as a transporter for a small molecule that modulates SMO activity is based on homology between PTCH and bacterial resistance, nodulation, division (RND) family of 12TM transporters [68], a family of antiporters that pump substrates out while importing protons down their concentration gradient (Fig. 6). The substrates of bacterial RND proteins range from metal ions to various xenobiotics, including sterol-like molecules called hopanoids [68]. Besides PTCH, Dispatched, Niemann-Pick C1 (NPC1) disease protein, NPC1-like 1 (NPC1L1) and the poorly characterized Patched-related (Ptr) proteins [69] are all eukaryotic members of the RND family. Further, all these proteins contain a five-transmembrane domain termed the sterol-sensing domain (SSD) that is conserved in three other proteins besides the RND family: the sterol sensor SCAP and two enzymes within the cholesterol biosynthetic pathway, HMG CoA reductase and DHCR7. Understanding how these proteins function may shed light on how PTCH regulates SMO.

Dispatched exports lipid-modified Hh proteins to the extracellular acceptor SCUBE [70,71]. NPC1 and NPC1L1 facilitate cholesterol transport across the lysosomal membrane and the intestinal epithelial cell apical membrane, respectively, in a direction that is topologically opposite to that of bacterial RND proteins. If NPC1 also uses a transmembrane proton gradient as an energy source, this would mean it acts as a symporter in contrast to its bacterial homologs. The N-terminal luminal domain of NPC1, which is distantly related to the SMO CRD [72], contains the initial binding site for cholesterol and receives cholesterol from the soluble luminal protein NPC2 and is postulated to deliver it to its SSD [73,74]. Consistent with this hypothesis, cryo-EM structures of NPC1 [75,76] revealed sterols within a tunnel that connects the N-terminal domain to the SSD. In contrast, yeast NPC1 and NPC2 homologs transport ergosterol, the primary sterol in yeast, which has a ring structure

identical to that of 7-dehydrocholesterol and was captured within a tunnel between the luminal domain and the SSD of yeast NPC1 [75,77].

SSDs found within paralogous as well as orthologous proteins can bind to different sterols and even phospholipids. In the case of SCAP, biochemical experiments have shown that cholesterol binds to a luminal loop rather than the SSD [78]. A recent cryo-EM structure, however, has shown that 25-hydroxycholesterol is sandwiched between the SCAP SSD and the 6TM protein Insig, and binds to the opposite surface of SSD compared to the sterol-like densities seen in PTCH1 structures [79]. In *Drosophila*, there is no Insig homolog, and the SCAP homolog is thought to sense phosphatidylethanolamine rather than sterols [80]. In mammals, Insig can also bind to the SSD within the regulatory domain of HMG CoA reductase in a sterol-dependent manner [81]. Although there is no direct evidence for sterol binding to HMG CoA reductase SSD, lanosterol and 24,25-dihydrolanosterol can induce Insig-dependent HMG CoA reductase degradation without affecting SCAP activity, suggesting differences in sterol specificity for binding to the two SSD proteins [82]. In the case of DHCR7, although the sequence similarity to SSDs of other proteins is low, the corresponding domain presumably serves as the binding site for the substrate 7-dehydrocholesterol.

A ‘Patched domain-containing protein’ called Ptchd3 was found to be localized to the sperm midpiece [83] but, as this gene is non-essential for viability and fertility [84,85], whether it is involved in regulating flagellar sterol composition is not clear. In *Caenorhabditis elegans*, there are 3 PTCH homologs and 24 Ptr proteins, which is perplexing because Hh pathway does not exist in this organism due to the absence of Hh and SMO orthologs [69]. Further, *C. elegans* does not synthesize cholesterol and only needs small amounts of it from dietary sources. Knockdown experiments revealed that these proteins function in multiple aspects of *C. elegans* development [86] but it is unlikely that all of them transport cholesterol. It remains to be seen if they transport different lipids, or act in different intracellular locations or cell types.

Several structures of truncated PTCH1 variants alone or in complex with either recombinant (unmodified) or native (lipid-modified) Shh have been determined by cryo-EM [87–91]. Despite significant differences between their oligomeric states or binding interfaces between PTCH1 and Shh, these structures support a model in which PTCH controls the accessibility of a sterol (perhaps cholesterol itself) to SMO, as revealed by sterols identified within a putative hydrophobic tunnel between its extracellular domains and SSD [13,22], similar to the tunnel found in mammalian and yeast NPC1 homologs. PTCH can accommodate sterols other than cholesterol as revealed by cryo-EM structures that show bound cholesterol hemisuccinate or the steroidal detergent glyco-diosgenin with two relatively large maltose moieties [92,93]. Regardless of the exact transport mechanism of PTCH, binding of Shh may inhibit the transport activity of PTCH. In support, the palmitate and cholesterol moieties of native Shh bind to the extracellular domains of PTCH1 in such a way to either prevent the binding of a PTCH1 substrate or block its movement through the tunnel [13]. In particular, the palmitate moiety occupies a site similar to one in NPC1 occupied by the small molecule inhibitor itraconazole [76]. However, earlier biochemical data indicated that unmodified and lipid-modified Shh can bind PTCH with the same

affinity even though palmitoylation dramatically increases Shh *potency* in most functional assays [94]. Surprisingly, structures of PTCH1 in complex with unmodified Shh showed that PTCH1 binds to the opposite surface of Shh when compared to lipid-modifed Shh. In fact, one lipid-modified Shh molecule can bind two PTCH1 molecules using the two different interfaces [89,90]. Although inhibition of Shh palmitoylation has dramatic effects on embryonic development, two observations suggest that the binding mode of unmodified Shh may also be physiologically relevant. First, recombinant (unmodified) Shh induces the differentiation of floor plate cells and motor neurons in chick neural plate explants with the same potency (2-25 nM) as native Shh [95]. Second, in cultured cell assays where unmodified Shh is inactive, introducing two hydrophobic isoleucine residues to its N terminus results in a substantial increase in its potency [96]. This Shh variant still binds PTCH1 in a manner identical to unmodified Shh [87]. It is possible that this binding mode still inhibits the transport activity of PTCH without the palmitate group penetrating deep inside the putative tunnel, but this would not explain why unmodified Shh can inhibit PTCH only in some contexts but not others. A more intriguing possibility is that PTCH may have two distinct activities depending on the cell type or developmental context (see below), that are inhibited by the two distinct binding modes of Shh. In summary, several structures of PTCH and its homology to RND proteins and NPC1 suggest that it functions, at least in part, as a transporter regulating the levels of a sterol lipid, but further work is necessary to uncover the identity of the substrate and the direction of transport. In this regard, PTCH1 was recently shown to use the transmembrane potassium gradient instead [97], but it remains unclear whether PTCH1 acts as a symporter or an antiporter.

SMO regulation likely evolved from a FZD-like mechanism where it was regulated by an extracellular ligand (a lipid or lipidated protein) that directly bound to its CRD to its current state, where the ability of SMO CRD to bind sterols can be seen as auxiliary, if not vestigial [98]. It is important to note that *Drosophila* SMO (dSmo) is unresponsive to cyclodextrin, sterols, and all synthetic ligands of mammalian SMO, and importantly, the structure of its CRD does not display a pocket that can accommodate sterols [99]. When dSmo was targeted to cilia by fusing it with the mammalian SMO C-terminal tail, the resulting chimeric protein was constitutively active suggesting that mammalian PTCH cannot repress dSmo [21]. This construct was also uninhibited by sterol depletion, consistent with the idea that dSmo is not regulated by a sterol agonist. Surprisingly, however, human PTCH1 expressed in fly cells can suppress dSmo activity as monitored by posttranslational modifications of downstream components [100]. One possible explanation for this discrepancy is that dSmo is regulated by an antagonist that does not exist in mammalian cells; but mammalian PTCH is more promiscuous and can transport this putative dSmo antagonist when expressed in fly cells. If the proposed model of PTCH function as a transporter is conserved in evolution, the same endogenous regulator of SMO would seem likely to operate in both vertebrate and fly systems because divergence of the effector small molecule would require the unlikely co-evolution of substrate specificity for PTCH transport and of binding specificity for SMO. It would be interesting to see if *Drosophila* Ptc targeted to cilia in mammalian cells could suppress mammalian SMO. Structure-function analyses, however, suggest that there may be fundamental differences in PTCH functions between flies and vertebrates. For instance, mutations in SSD confer dominant negative activity to *Drosophila* Ptc but

does not detectably alter the function of mammalian PTCH [101]. Similarly, the poorly conserved cytoplasmic C-terminal domain (CTD) of Ptc is essential in *Drosophila* and its deletion creates a dominant-negative protein that blocks wild-type Ptc function *in vivo* [102]. In contrast, the CTD of mammalian PTCH1, which is deleted in the constructs used for all published structural investigations, seems to be required in a tissue-specific manner. This was revealed by a spontaneous mouse mutant called mesenchymal dysplasia (*mes*) caused by a deletion of part of the CTD [103]. On the one hand, *mes* homozygotes exhibit normal spinal cord development in contrast to *Ptch1*$^{-/-}$ homozygotes, which die around day 10 of embryonic development with severe neural tube defects [104]. This indicates that CTD is dispensable in early embryogenesis. On the other hand, *mes* mutants exhibit pleiotropic Hh-associated phenotypes including excess skin, increased body weight and preaxial polydactyly. These observations support the idea that, depending on the tissue or cell type, PTCH1 may suppress SMO activity by two different mechanisms, one that is CTD-dependent and another that is CTD-independent, where *Drosophila* Ptc mainly uses the first mechanism. Another potential difference between *Drosophila* and vertebrate systems was revealed with the discovery of a negative regulator of the *Drosophila* Hh pathway, called Target of Wingless (Tow), that seems to act between Ptc and Smo [105]. No such factor has been identified in vertebrates.

5. Oncogenic Hh signaling: SMO on steroids

Dysregulated Hh signaling is known to drive multiple cancers. This was first appreciated through patients with Gorlin's syndrome, a rare genetic condition caused by heterozygous inactivating mutations in negative regulators of the Hh pathway, who are predisposed to developing basal cell carcinomas (BCCs), medulloblastomas, and rhabdomyosarcomas (RMSs) [106–108]. Hh pathway misactivation also underlies sporadic BCCs, the most common cancer in the United States [109–111], approximately one-third of sporadic medulloblastomas, one of the most common malignant brain tumors in children [112,113], and a subset of sporadic RMSs, the most common pediatric soft tissue sarcoma [114–116]. Aberrant Hh signaling has been also implicated in several other malignancies, including pancreatic ductal adenocarcinoma, lung cancer, and prostate cancer, but the mechanistic connections linking Hh signaling to these tumors are incompletely understood [6]. Understanding how Hh signaling drives tumor growth across cancer subtypes is fundamental, as small molecules modulating the Hh pathway represent a powerful therapy modality.

In this regard, several synthetic small-molecule inhibitors of the Hh pathway have been developed and are approved for use in cancer patients, including the SMO inhibitors vismodegib and sonidegib. These inhibitors have been extensively studied in the treatment of medulloblastoma and BCC [117–119], leading the FDA approval of vismodegib and sonidegib for metastatic or locally advanced BCC, and vismodegib for use in recurrent medulloblastoma. Responses to SMO inhibitors are variable, and resistance to molecular monotherapy is common in Hh-associated cancers. Both BCC and medulloblastoma can regress below the threshold of detection by visual, histopathological, or radiological examination, but many tumors can recur after developing resistance mechanisms [120]. Point mutations in SMO [121] and misactivation of the PI3K pathway [122] underlie

resistance to SMO inhibitors in Hh-associated medulloblastoma, and similar mutations in SMO [123] and non-canonical activation of the Hh pathway [124] underlies resistance to SMO inhibitors in BCC. Moreover, these therapies cause myriad adverse side effects, such as nausea, muscle cramps, loss of taste, weight loss, and alopecia, leading to medical non-compliance [125]. Pediatric medulloblastoma patients who are treated with SMO inhibitors also have short stature and other medical problems due to premature and irreversible growth plate fusion from systemic Hh pathway inhibition [126]. Considering the resistance mechanisms to Hh pathway inhibition in cancer, and the adverse side effects of these medications, there is an urgent, unmet need for new therapies to treat Hh-associated cancers.

With respect to modulating oncogenic Hh signaling for therapeutic purposes, naturally occurring oxysterols, including the antagonist DHCEO, have modest affinities to SMO that are typically in the micromolar range. There are also many other oxysterol receptors with diverse ligand-binding specificities that may cause pleiotropic effects, especially if a high concentration is needed to inhibit oncogenic Hh signaling. A different approach to inhibit Hh pathway would be to deprive SMO of its endogenous agonists. Given that both SMO and PTCH are likely capable of binding diverse sterols, it is possible that there is a different endogenous regulator of SMO depending on cell type, tissue, developmental or oncogenic context. In addition, overproduction of an abnormal oxysterol agonist of SMO by tumor cells can conceivably drive constitutive SMO activity. Regardless, there is a positive role for sterols for the activation of Hh pathway. Inhibition of HMG CoA reductase by statins indeed repress Hh signaling and proliferation in medulloblastoma cells [127]. Statins further synergize with vismodegib in inhibiting medulloblastoma growth. This is a promising strategy given that point mutations in SMO that could give rise to vismodegib resistance would not necessarily circumvent the positive requirement for sterols, and statins are used safely by millions of people as cholesterol-lowering drugs.

Recently, HSD11β2 was identified as a Hh target gene and a novel therapeutic target in Hh-associated medulloblastoma [11]. HSD11β2 is an oxysterol synthase that produces SMO-activating lipids to drive Hh signaling in development and medulloblastoma, and inhibition of HSD11β2 using a small molecule derived from black licorice, carbenoxolone, blocks the Hh pathway and the growth of Hh-associated medulloblastoma [128] (Fig. 4). Recently, HSD11β2 enzymatic activity was shown to be inhibited by the antifungal drugs itraconazole and posaconazole [129]. These drugs were originally known as inhibitors of lanosterol demethylase (CYP51) but have since been shown to also interfere with intracellular sterol trafficking, at least in part, by inhibiting NPC1 [130] (see above) and oxysterol-binding protein family [131]. A combination of these mechanisms can explain SMO inhibition observed with these drugs [132].

Moreover, recent work has identified that CDK6 is a direct transcriptional target of oncogenic Hh signaling, and CDK6 antagonists such as abemaciclib and palbociclib are effective treatments in preclinical models of Hh-associated medulloblastoma and are currently under investigation in clinical trials [128]. Nevertheless, genetically engineered mouse models of Hh-associated medulloblastoma treated with carbenoxolone or CDK6 inhibitors ultimately succumb to their tumors [11,128]. These data suggest that Hh-associated cancers can also develop resistance mechanisms to therapies blocking non-

canonical Hh pathway activators or effectors. Genome-wide CRISPR screens and transcriptomic profiling of Hh-associated medulloblastomas lacking *Cdk6* reveals loss of CDK6 leads to ER stress, activating the unfolded protein response and inducing enzymes producing SMO-activating lipids, such as HSD11β2 and DHCR7, that sustain oncogenic Hh signaling [133]. Consistently, combination molecular therapy with abemaciclib and carbenoxolone blocks the growth of Hh-associated medulloblastoma and prolongs survival more than molecular monotherapy [133]. Thus, preclinical strategies targeting lipids that activate the Hh pathway in conjunction with effectors of oncogenic Hh signaling overcome resistance mechanisms and may improve outcomes in patients with Hh-associated cancer.

6. Conclusion

The structures of PTCH1 and SMO provide insights into putative substrate/ligand binding sites and confirm the similarity between PTCH and other eukaryotic RND proteins, but they have not clarified the identity of endogenous substrates or ligands, or the direction of PTCH transport of those molecules. Future studies directed at elucidating endogenous lipids that bind PTCH and SMO would benefit from native mass spectrometry, which has emerged within the last decade as a complementary technique to probe protein-lipid interactions [134]. The exact mechanism of PTCH action will likely require reconstitution of its transport activity in a cell-free system. With respect to Hh signaling and cancer, multiple open questions remain that will require further investigation: (1) What other human malignances are driven by misactivated Hh signaling? (2) Why do some tumors respond well to targeted Hh inhibitors, whereas other tumors rapidly develop resistance to monotherapy? (3) Will oxysterol synthase inhibitors, such as carbenoxolone translate to the clinic? Answering these questions will not only further our understanding of Hh signaling and cancer but will also lead to improved cancer therapies.

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Abbreviations

BCC	basal cell carcinoma
ChEH	cholesterol-5,6-epoxide hydrolase
Ci	Cubitus interruptus
CRD	cysteine-rich domain
CTD	C-terminal domain
cryo-EM	cryo-electron microscopy
Cyp	cytochrome P450
DHCEO	3β,5α-dihydroxycholest-7-en-6-one
DHCR7	7-dehydrocholesterol reductase

DHCR24	24-dehydrocholesterol reductase
dSmo	*Drosophila* Smoothened
EBP	emopamil-binding protein
ECD	extracellular domain
ER	endoplasmic reticulum
FZD	Frizzled
GPCR	G protein-coupled receptor
Hh	Hedgehog
HMG CoA	3-hydroxy-3-methylglutaryl coenzyme A
HSD11β2	11β-hydroxysteroid dehydrogenase type 2
MCD	methyl-β-cyclodextrin
mes	mesenchymal dysplasia
NPC1	Niemann-Pick C1
NPC1L1	Niemann-Pick C1-like 1
PE	phosphatidylethanolamine
PFO	perfringolysin O
Ptc	Patched
PTCH	Patched homolog
Ptchd	Patched domain
Ptr	Patched-related
RMS	rhabdomyosarcoma
RND	resistance, nodulation, division
SAG	Smoothened agonist
SLOS	Smith-Lemli-Opitz syndrome
SMO	Smoothened
SREBP	sterol regulatory element-binding protein
SSD	sterol-sensing domain
Tow	Target of Wingless
7TM	seven-transmembrane

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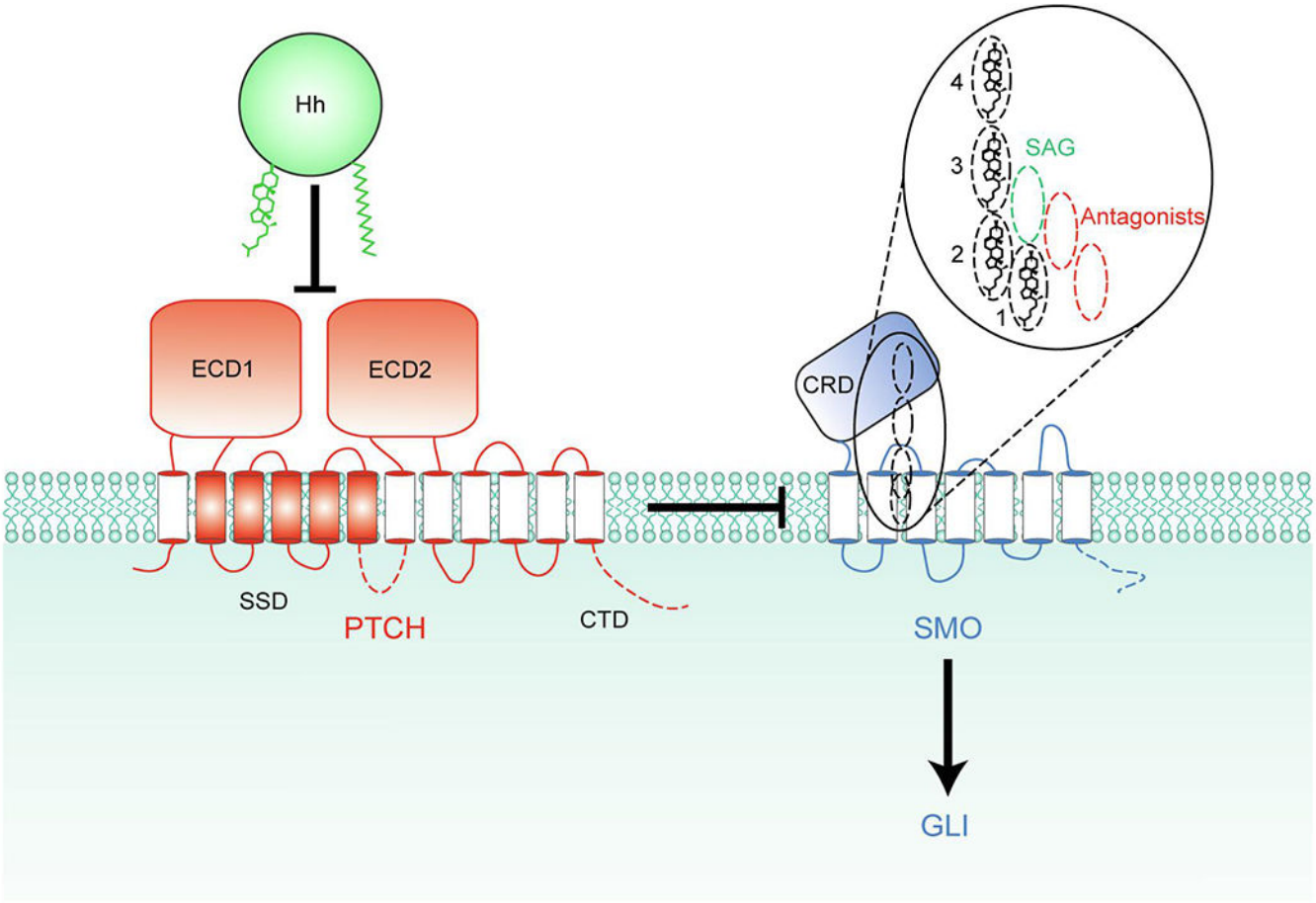

Fig. 1.

PTCH inhibits SMO by an unknown mechanism. Binding of Hh inhibits PTCH, allowing SMO activation. The 4 partially overlapping sites within SMO that can be occupied by sterols are depicted as dashed ovals, and their vertical positions compared to the synthetic SMO agonist (SAG) and various antagonists are shown in the inset. Sterols in all 4 sites within SMO have the same orientation (hydroxyl group facing towards the extracellular side and the side chain facing towards the intracellular side) potentially allowing sterols to move between these sites without flipping. The intracellular domains of PTCH1 and SMO missing from the structures, including the C-terminal domain (CTD) of PTCH1, are indicated by dashed lines. ECD1 and ECD2, extracellular domains 1 and 2; SSD, sterol-sensing domain; CRD, cysteine-rich domain.

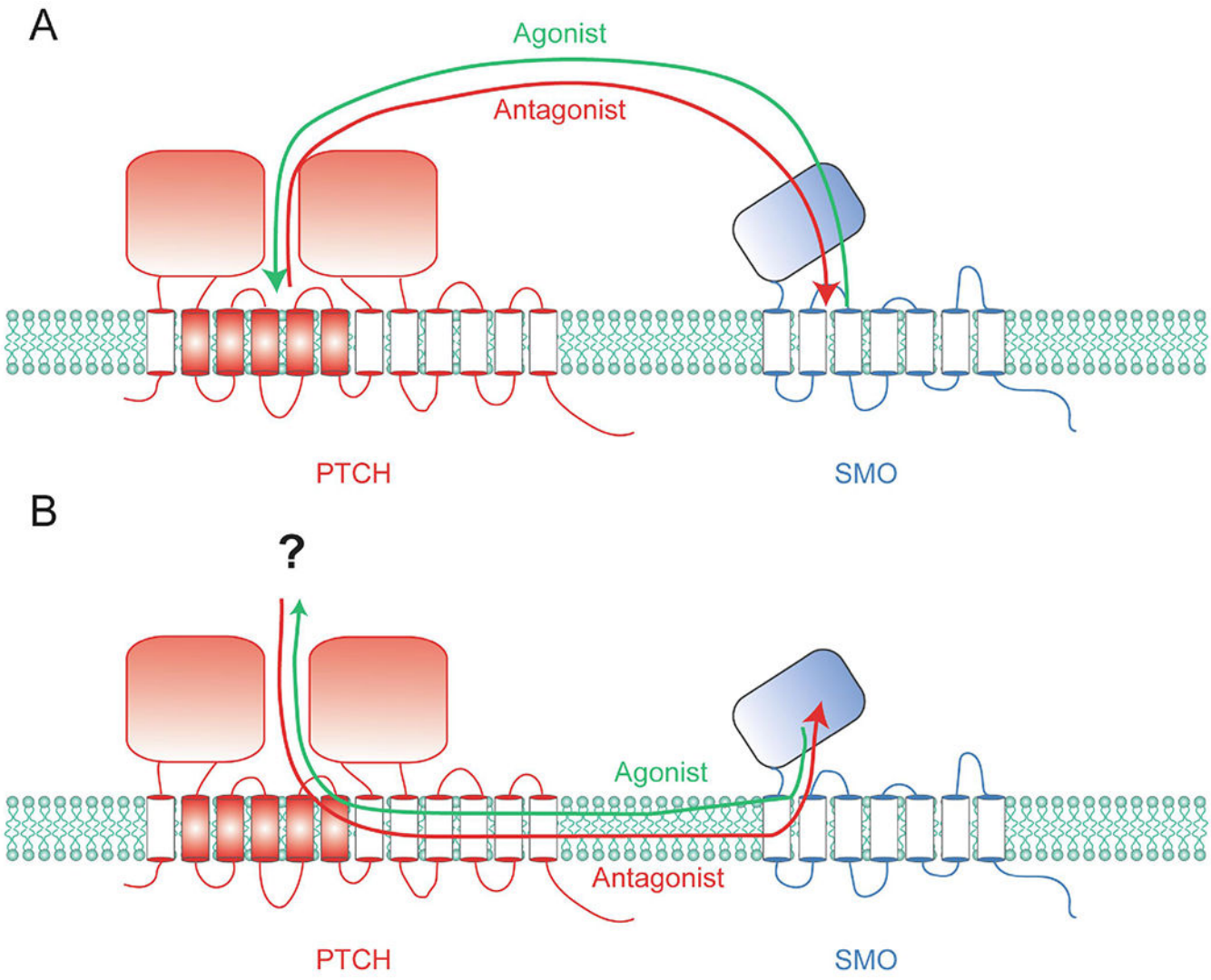

Fig. 2.

Models for PTCH regulation of SMO. PTCH likely inhibits SMO by either removing an agonist from SMO (green arrows) or transferring an antagonist to SMO (red arrows). It is unclear whether the exchange between PTCH and SMO occurs between the extracellular domains (A) or transmembrane domains (B). The latter case implicates the existence of a sterol carrier protein that cooperates with PTCH (shown with a question mark).

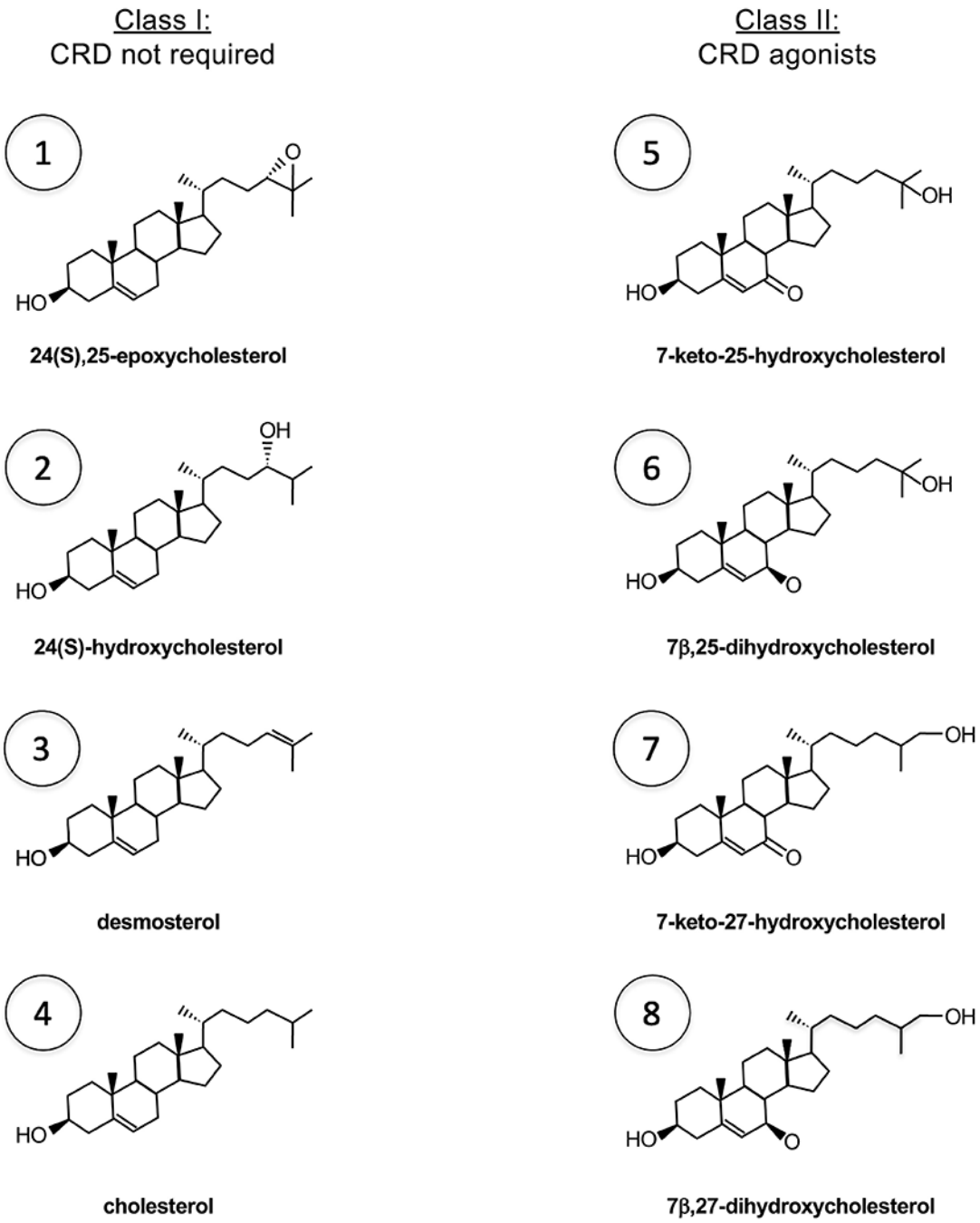

Fig. 3.

Sterol agonists of SMO. Class I sterols do not require CRD as they can activate SMOΔCRD when delivered as MCD inclusion complexes, although cholesterol and 24(S),25-epoxycholesterol can also bind CRD. Class II sterols only bind SMO CRD and can activate full-length SMO but not SMOΔCRD regardless of whether they are added from ethanol stocks or as MCD complexes. When a class I and a class II sterol are added together from ethanol stocks, a synergistic (more than additive) effect is observed. See Fig. 4 for biosynthesis of individual sterols (indicated by numbers).

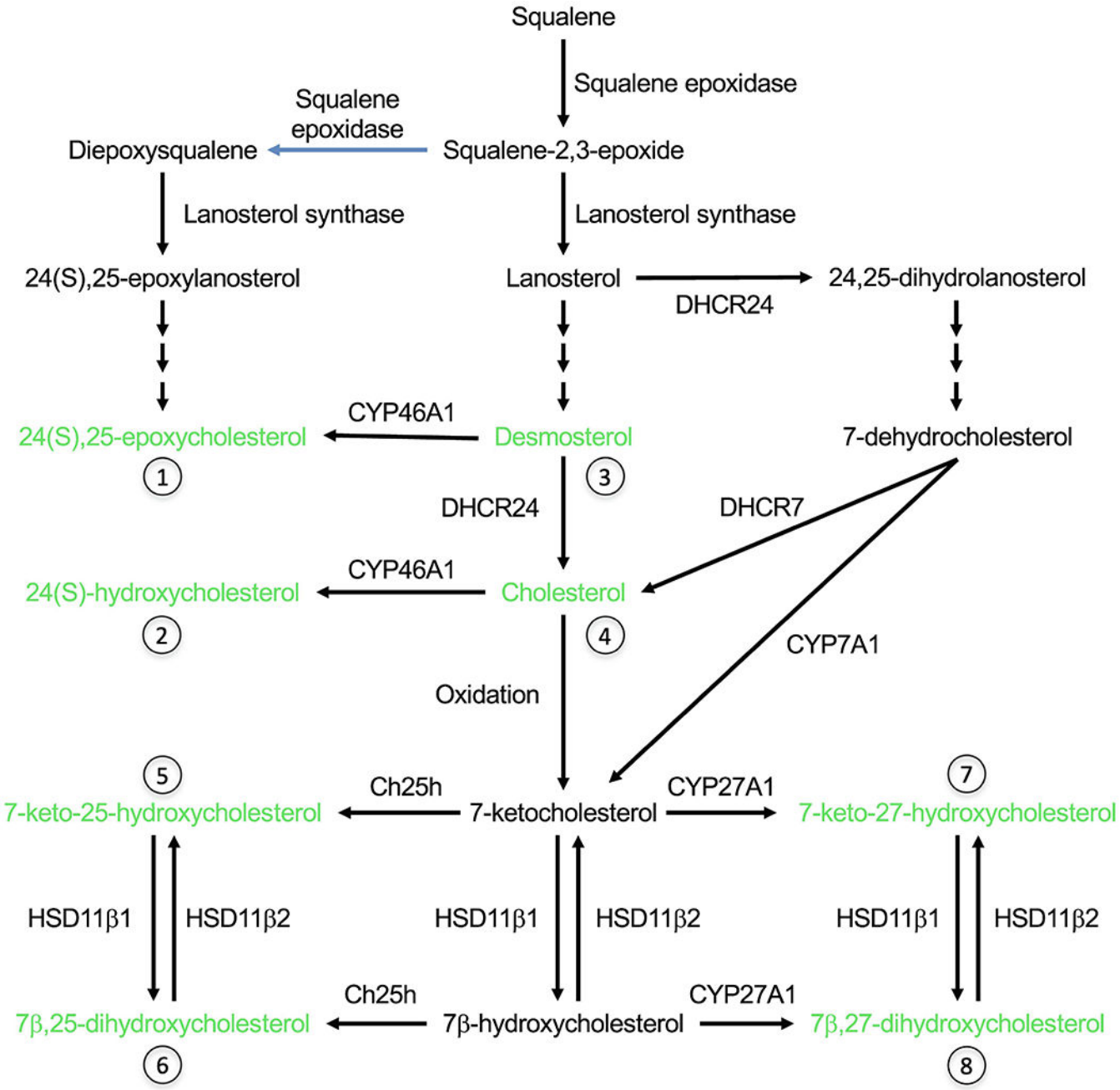

Fig. 4.

Biosynthesis of cholesterol and the sterol agonists of SMO (shown in green). 7-ketocholesterol can be formed either by non-enzymatic oxidation of cholesterol or enzymatically from 7-dehydrocholesterol. See Fig. 3 for the structures of sterols indicated with numbers.

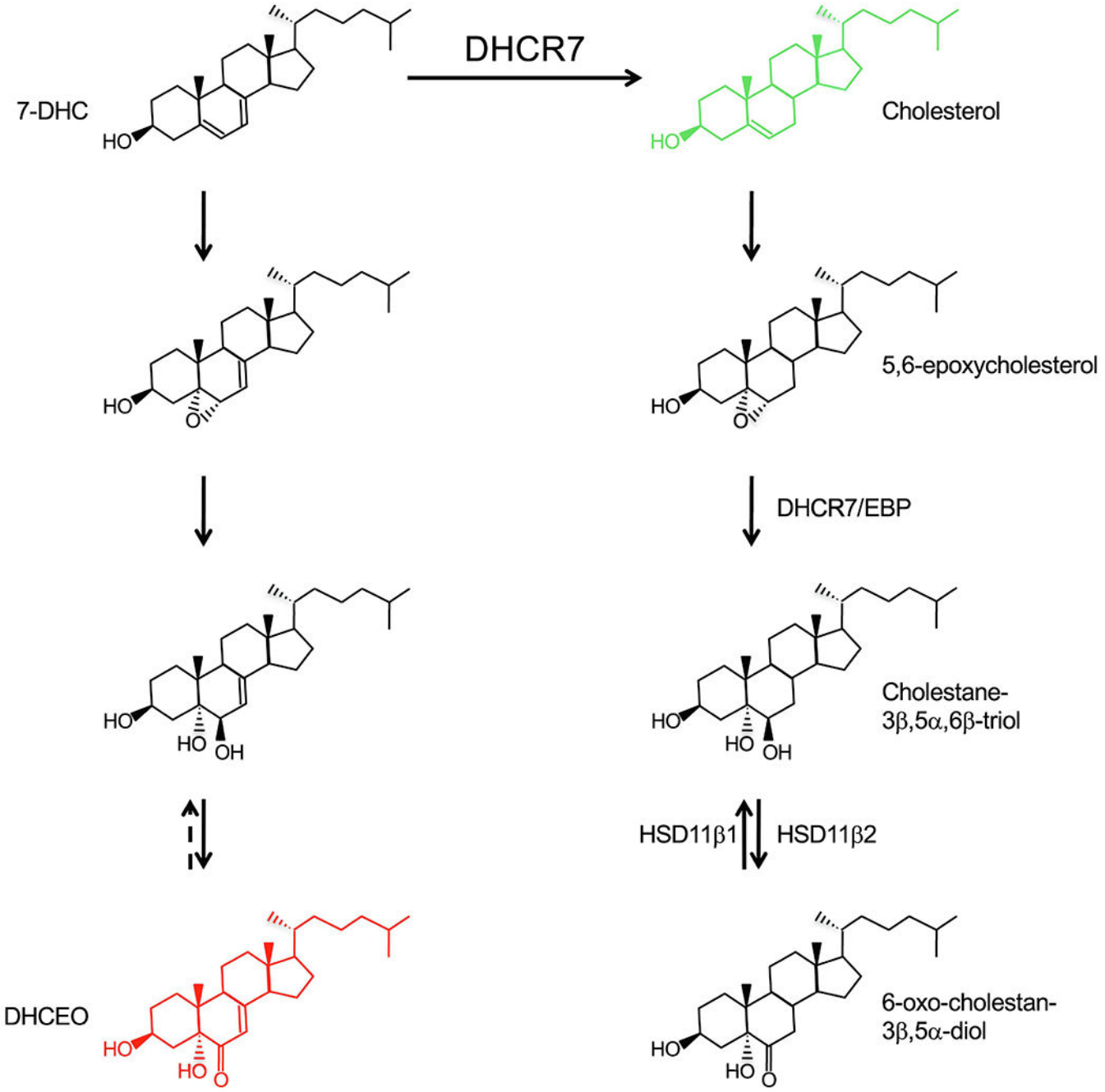

Fig. 5.

Formation mechanism of the SMO antagonist DHCEO. Besides cholesterol, which has a positive effect, none of the other sterols shown affect Hh pathway (unpublished observations). Whether the last step of DHCEO synthesis is reversible and which enzymes are responsible are not known.

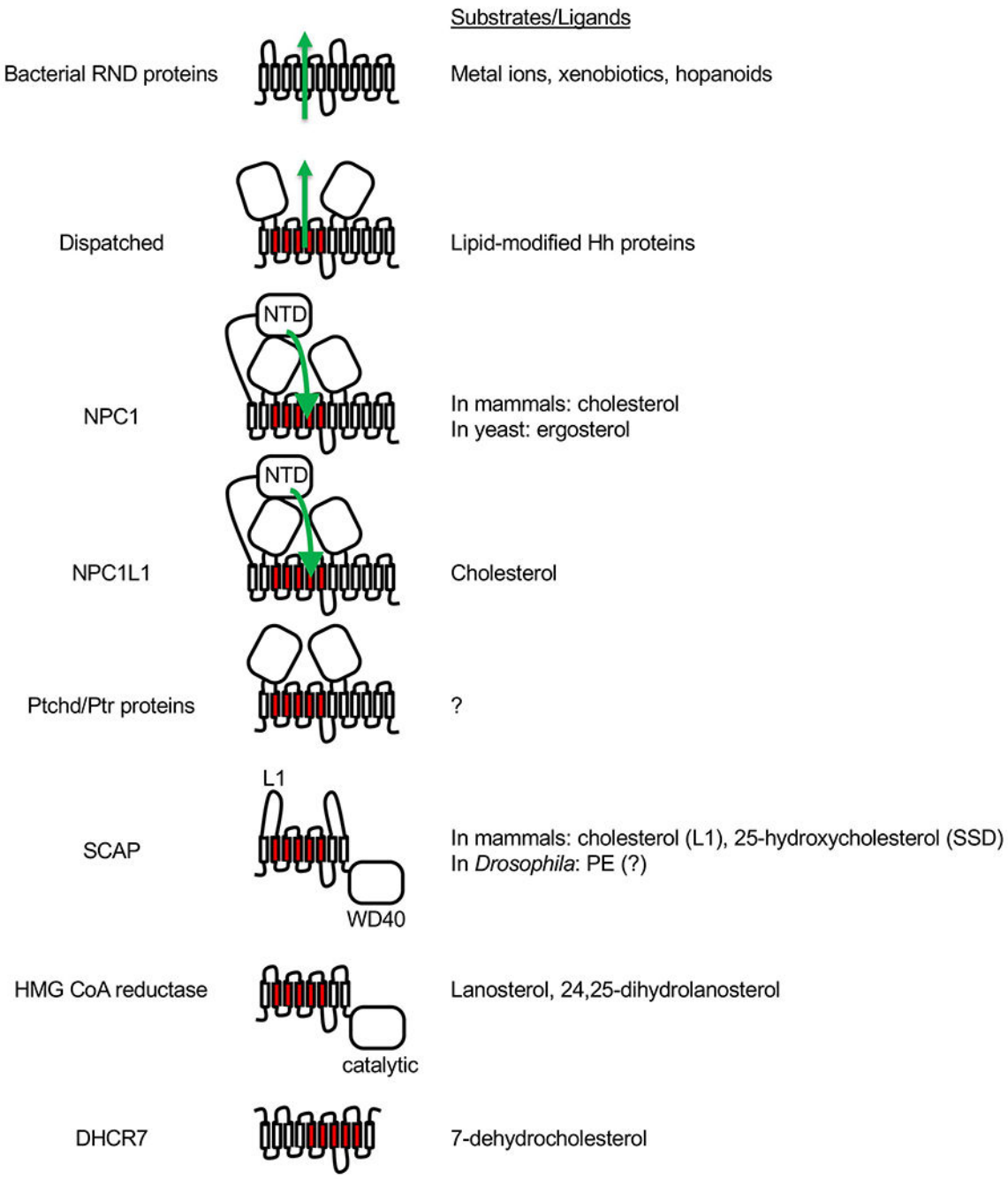

Fig. 6.

Bacterial RND proteins and eukaryotic SSD family members. For all proteins, the extracellular or luminal side faces up, and the cytosolic side faces down. The five TM domains that constitute the SSD are shown in red. Dispatched, NPC1, NPC1L1 and Ptchd/Ptr proteins are thought to function as transporters similar to the bacterial RND proteins, and the direction of substrate transport (where known) is indicated with green arrows. NPC1 and NPC1L1 each has an additional N-terminal domain (NTD). In the case of NPC1, cholesterol or ergosterol is first transferred from the luminal NPC2 protein (not

shown) to the NTD and then travels through a tunnel between the other two large luminal domains towards the SSD. SCAP and HMG CoA reductase each has 8 TM domains and an additional cytosolic domain. In the case of SCAP, the cytosolic WD40 domain binds sterol regulatory element-binding proteins (SREBPs). Binding of either cholesterol to loop 1 (L1) or of 25-hydroxycholesterol to SSD of mammalian SCAP causes retention of SCAP-SREBP complex in the ER. *Drosophila* SREBP pathway responds to phosphatidylethanolamine (PE) instead of sterols but direct binding of PE to *Drosophila* SCAP has not been demonstrated. In the case of HMG CoA reductase, the cytosolic domain is necessary and sufficient for its catalytic activity, which is inhibited by statins. The membrane domain mediates ER-associated degradation of HMG CoA reductase in the presence of excess sterols. Lanosterol and 24,25-dihydrolanosterol have been shown to induce HMG CoA reductase degradation without affecting SREBP pathway. The topology of DHCR7 is not certain as it is predicted to contain 9 or 10 TMs, but its SSD is presumably the catalytic site where 7-dehydrocholesterol binds as a substrate.

Table 1

Arguments for and against different models by which PTCH regulates SMO.

Model1: PTCH reduces accessibility of (ciliary) cholesterol for SMO
Pro: Unless a different sterol or steroidal detergent is added during purifications, the sterol-like densities seen in PTCH1 and SMO structures are likely cholesterol.
Con:
- Alteration of cholesterol distribution by PTCH would have pleiotropic effects.
- SMO activity would be affected by various other cholesterol transporters.
- An ultimate acceptor for cholesterol removed away from SMO is required.
Model 2: PTCH removes an oxysterol agonist from SMO
Pro: specific
Con:
- affinity << abundance (except 24(S),25-hydroxycholesterol, see text)
- no genetic evidence (does not apply for 24(S),25-hydroxycholesterol, see text)
Model 3: SMO requires cholesterol but PTCH inhibits SMO via a sterol antagonist
Pro:
- specific
- evidence for a positive effect of MCD under certain conditions
- can explain non-cell-autonomous inhibition of SMO by PTCH
Con: hard to genetically disentangle a negative effect of 7-dehydrocholesterol derivatives from the positive effect of cholesterol

Vikas Daggubati: Writing- Original draft preparation, Writing- Reviewing and Editing. **David R. Raleigh**: Writing- Reviewing and Editing. **Navdar Sever**: Conceptualization, Writing- Original draft preparation, Writing- Reviewing and Editing, Supervision.