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Multiple ligand binding sites regulate the Hedgehog signal transducer Smoothened in vertebrates

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The Hedgehog (Hh) pathway plays a central role in the development of multicellular organisms, guiding cell differentiation, proliferation and survival. While many components of the vertebrate pathway were discovered two decades ago, the mechanism by which the Hh signal is transmitted across the plasma membrane remains mysterious. This fundamental task in signalling is carried out by Smoothened (SMO), a human oncoprotein and validated cancer drug target that is a member of the G-protein coupled receptor protein family. Recent structural and functional studies have advanced our mechanistic understanding of SMO activation, revealing its unique regulation by two separable but allosterically-linked ligand-binding sites. Unexpectedly, these studies have nominated cellular cholesterol as having an instructive role in SMO signalling.

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Introduction

The Hedgehog (Hh) signalling pathway is essential for embryogenesis and adult stem cell homeostasis in all bilaterians [1–3]. Its dysregulation is linked to developmental abnormalities and various types of cancers, including basal cell carcinoma (BCC) and medulloblastoma. Many tissues in the early embryo are patterned by gradients of morphogens, exemplified by ligands such as Hh in *Drosophila* and Sonic Hedgehog (SHH) in vertebrates. Local concentrations of such morphogens are interpreted by target cells to drive cell fate decisions, ultimately forming the basis for a body plan.

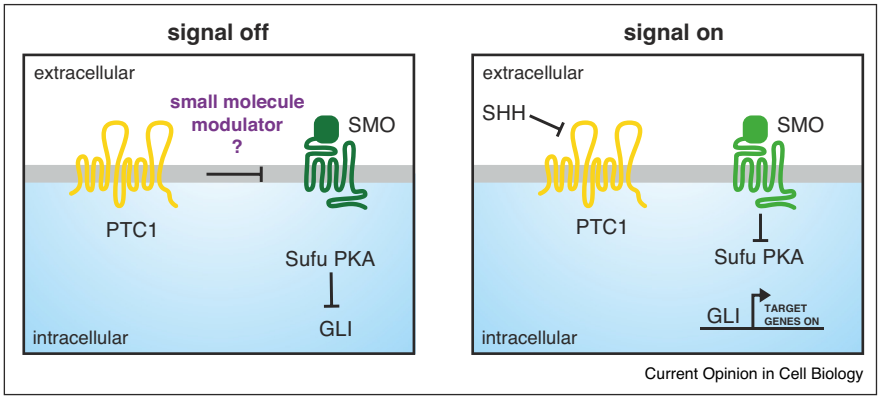
In Hh-producing cells, a precursor form of a secreted Hh ligand (e.g. SHH) is expressed and auto-catalytically cleaved into a doubly lipidated N-terminal domain to produce the mature morphogen [1–3]. Hh ligands are received on target cells by the twelve-pass transmembrane (TM) protein Patched 1 (PTC1), in cooperation with other co-receptors that can modify ligand reception (for details see [4–6]). A unique feature of the Hh signalling cascade is that ligand reception and signal transduction across the plasma membrane have been assigned to two different membrane proteins, PTC1 and Smoothened (SMO). SHH binding inactivatesPTC1, thereby relieving its constitutive inhibition of the G-protein coupled receptor (GPCR) SMO (Figure 1). SMO activation is the critical step that transmits the Hh signal across the membrane to the cytoplasm, ultimately resulting in the activation of the glioma-associated oncogene family members (GLI) transcription factors [1,2]. This separation of function requires that the Hh signal must be relayed from PTC1 to SMO, a mysterious step in signalling that is thought to be mediated by a small molecule second messenger [7–9] (Figure 1).

SMO has been the focus of intense study because it is required for transmembrane signalling in all animals and because it has become a validated drug target for Hh-driven human cancers [3,10]. This review will discuss recent advances and enduring puzzles related to the mechanisms of SMO signal transduction, with a focus on the recent structural characterization of SMO and its regulation by various small molecules.

Architecture of Smoothened

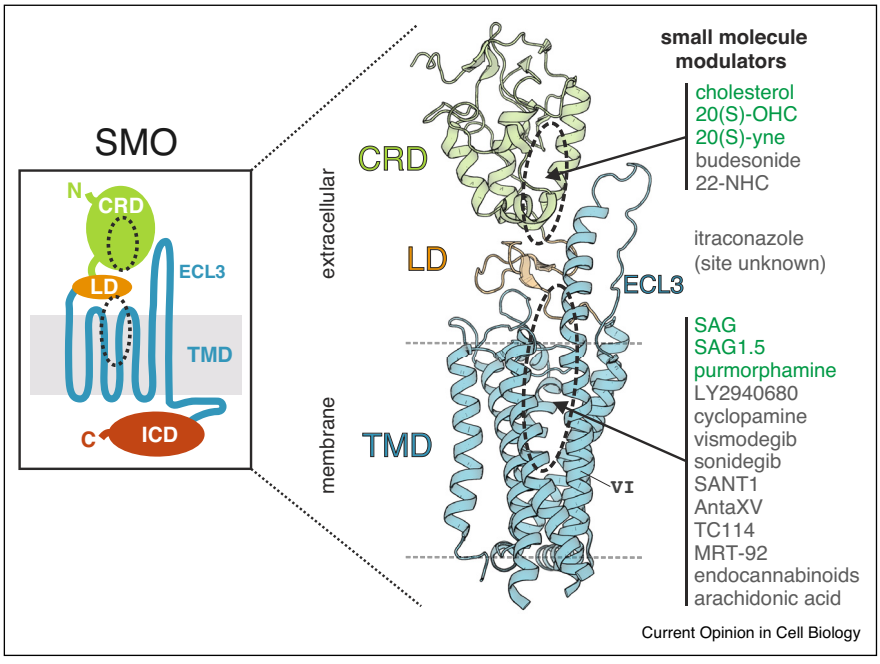
SMO is a Frizzled-class GPCR. Unlike the 'classical' Class A GPCRs, SMO contains not just the stereotypical seven-pass α-helical transmembrane bundle (TMD), but also sizeable extracellular and intracellular domains (Figure 2, left panel). The extracellular region of SMO consists primarily of a cysteine-rich domain (CRD), named for its conserved disulphide bonding architecture, which has homologs in the Frizzled receptors (Fzd), Niemann–Pick type-C protein 1 (NPC1), and riboflavin-binding protein [11]. The CRD is connected to the TMD through a short linker domain (LD), itself containing a disulphide bond. The intracellular domain of SMO is partly unstructured and has been shown to be important

Figure 1



PTC1 regulates SMO via an unknown mechanism. In the absence of SHH, PTC1 inhibits SMO, which allows Sufu and PKA to inhibit the GLI transcription factors (left panel). In the presence of SHH, PTC1 releases its inhibition of SMO, which in turn is able to signal downstream, ultimately resulting in the transcription of target genes by GLI (right panel).

Figure 2



The overall structure of SMO. SMO consists of a large extracellular region, made up of the CRD (green) and LD (orange), and an intracellular domain (ICD, red) in addition to the seven-pass α-helical transmembrane domain (TMD, blue) (left panel). The multi-domain SMO crystal structure revealed a stacked domain arrangement with two physically separable binding sites (right panel). The approximate location of the two binding sites is marked with dashed black ovals in both left and right panels. ECL3 and TMD helix VI are also labeled. Agonist (green) and antagonist (dark grey) small molecule modulators are listed on the right and associated with a particular binding site, if known. This list is not exhaustive.

for SMO localisation in primary cilia and subsequent downstream signalling.

While structures of the isolated CRD and TMD of SMO have been previously reported and reviewed [12–16], more recent structural studies of a multi-domain SMO protein have revealed the critical interactions between these domains and how they may be modulated by small molecules [17••,18••]. These new structures show a stacked domain arrangement with the CRD perched atop the TMD with an intervening wedged LD. The extracellular extension of TMD helix VI, part of extracellular loop 3 (ECL3), enables the TMD to interact directly with the CRD (Figure 2). This arrangement suggests that signal transmission from the CRD to the TMD is mediated by the LD and ECL3. Contacts between the CRD,

LD and TMD seem to be critical for stabilizing the inactive state of vertebrate SMO; complete deletion of the CRD increases the constitutive signalling activity of SMO, as do mutations designed to destabilize the LD or contacts between the LD and the CRD [17••].

Several limitations of these recently reported structures are worth highlighting. First, the SMO structures solved to date do not give any insights into TMD conformational changes associated with activation or insights into how SMO transmits signals to the cytoplasm. Indeed the TMD domains in the SMO structures almost look identical, regardless of whether they are bound to antagonists or agonists [17••,18••,19]. The multi-domain SMO proteins used in these recent structural studies are incapable of coupling to cytoplasmic signalling components for at least three reasons: they lack the C-terminal tail, they contain stabilizing heterologous proteins inserted into the third intracellular loop (critical for the signalling function of many GPCRs) and their TMDs are locked in an inactive state by either mutations or high-potency antagonists.

Small molecule modulators of Smoothened

Unlike many GPCRs, SMO can be activated or inhibited through at least two ligand-binding sites (reviewed in [15]). The first binding site (hereafter the 'TMD site'), which corresponds to the canonical 'orthosteric' ligand-binding site in many GPCRs, is located at the extracellular end of the transmembrane bundle (Figure 2, right panel). The TMD site was first shown to engage the SMO antagonist cyclopamine [20] and subsequently shown to bind to multiple small molecule agonists and antagonists, including the two high-potency inhibitors vismodegib and sonidegib used to treat advanced BCC in the clinic [3,15,21–27]. Pioneering structures of the isolated TMD showed that TMD ligands bind at various depths within a vestibule at the extracellular end of the TMD and revealed how mutations in residues lining this vestibule can lead to resistance to SMO antagonists used in the clinic [12,19]. The second site, formed by a shallow hydrophobic groove on the surface of the CRD, can bind and mediate the effects of oxysterols and cholesterol on SMO activity [13,17••,28,29,30,31••,32••] (Figure 2, right panel).

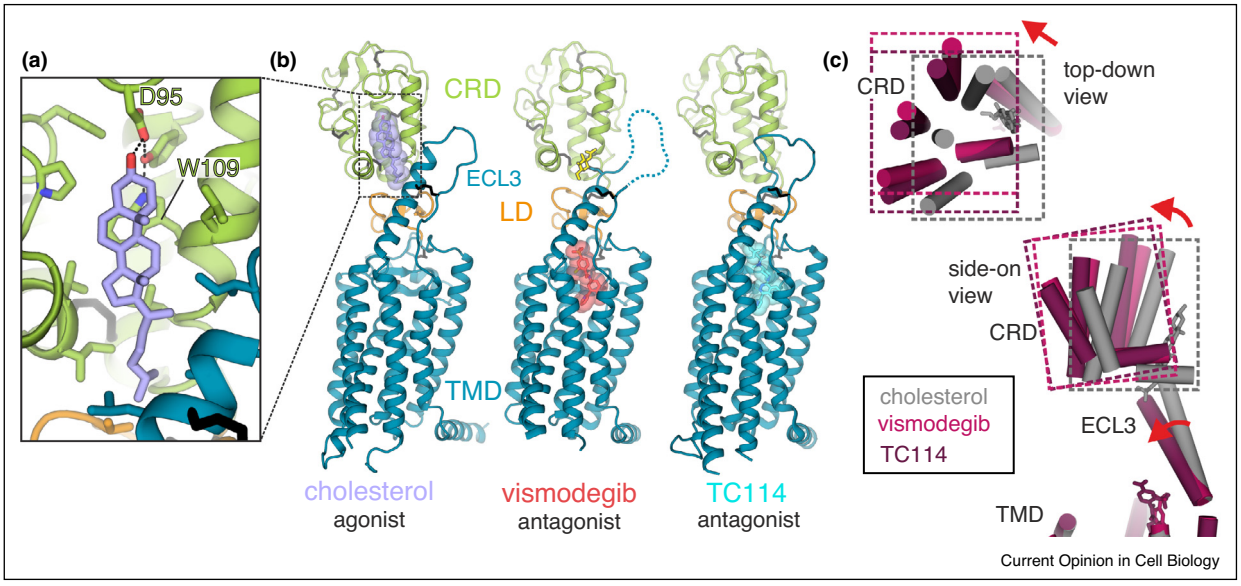
While these two ligand-binding sites are housed in distinct, physically separable domains, early pharmacological studies suggested that they were allosterically linked [28]. A structural basis for this communication was revealed by multi-domain crystal structures of SMO bound to cholesterol in the CRD site or two high-potency antagonists, vismodegib or TC114, in the TMD site [17••,18••]. Cholesterol was recently shown to be a direct SMO agonist that can bind to the CRD in solution and is sufficient to activate signalling even in the absence of Hh ligands [31••,32••]. In the multi-domain structure, the cholesterol molecule fills the hydrophobic CRD binding groove, forming essential interactions with several residues including Asp95 and Trp109, and is partially shielded from the solvent by ECL3 (Figure 3a) [17••]. The CRD binding site and the cholesterol molecule within it are positioned at the intersection of the CRD, LD and TMD, ideally located to mediate interactions between the three domains that could regulate SMO activity (Figure 3b, left panel). A recent study suggested that the 3β-hydroxyl of cholesterol can form a covalent ester bond with Asp95 [33••], though this was not evident in the high-resolution structure [17••]. While the CRD and TMD binding sites are separated by ~12 Å, vismodegib and TC114 binding to the TMD alters the orientation of the CRD, LD and ECL3 relative to the TMD, resulting in displacement of cholesterol from the CRD ligand-binding groove [17••,18••] (Figure 3b, middle and right panels, Figure 3c). The CRD binding site is occluded by a sugar moiety in the vismodegib-bound structure or by sidechains of several residues from ECL3 in the TC114-bound structure (Figure 3b). Thus, the observed allosteric linkage between the two sites is likely to be facilitated by structural communication mediated by ECL3 and the LD [17••,28] (Figure 3c).

In addition to the TMD and CRD sites noted above, the C-terminal tail of SMO has been proposed to mediate the potentiating effect of phosphatidylinositol 4-phosphate (PI(4)P) on SMO activity, but we lack any structural information on the C-tail or pharmacological information on how this interaction might influence the CRD and TMD ligand binding sites [34,35].

Ligand-binding sites that regulate endogenous SMO signalling

Much of our understanding of SMO function described above comes from studies of signalling in response to exogenously added ligands. But what are the endogenous ligands that regulate SMO activity and do they act through the CRD site, the TMD site or a yet undiscovered third site? Both the CRD and TMD sites have been subjected to mutagenesis to address this question. Mutations in the CRD site can impair signalling, not just in response to oxysterols and cholesterol, but also in response to SHH (which does not act directly on SMO but rather indirectly through PTC1 (Figure 1)) [31••,32••]. The importance of the CRD-site for endogenous signalling was highlighted by the demonstration that a point mutation in Asp95 (which makes a critical hydrogen bond with cholesterol in the SMO structure (Figure 3a)) impairs mouse embryonic development when knocked into the endogenous *smo* locus [33••], closely phenocopying a null *smo* allele. In addition, a complete deletion of the CRD (SMO-ΔCRD) or more subtle mutations that disrupt CRD-LD interactions, resulted in SMO molecules with elevated, ligand-independent constitutive activity [17••,29,30]. SMO-ΔCRD is markedly less sensitive to PTC1 and nearly fully activated, consistent with the model that the CRD site

Figure 3



Multi-domain structures of SMO. **(a)** Close-up of the cholesterol binding site in the SMO CRD. Residues involved in binding are shown as sticks. Dotted black lines indicate potential hydrogen bonds. Two important residues also discussed in the text are labeled (Asp95 and Trp109). **(b)** Three multi-domain structures of human SMO, each solved with a different ligand (as indicated beneath each structure), are shown in the same orientation (PDB: 5L7D [17••], 5L7I [17••], 5V57 [18••]). The glycan occluding the CRD-site in the vismodegib complex (middle) is shown in yellow stick representation. Domains in **(a)** and **(b)** are coloured as in Figure 2. **(c)** Conformational changes associated with antagonist binding result in collapse of the CRD binding site, thus precluding cholesterol binding. The three multi-domain structures of SMO were aligned by their TMDs. Each structure is coloured separately with helices shown as solid cylinders and loops omitted for clarity. Red arrows indicate domain movements between structures.

plays an important role in mediating the inhibitory influence of PTC1 on SMO [32••].

In contrast to CRD mutations, several mutations introduced into the TMD site failed to alter either basal or SHH-regulated SMO activity in cultured cells, even though they abrogated the effects of synthetic TMD ligands [36,37]. Thus, the TMD site in SMO that corresponds to the main regulatory site in other GPCRs, may only play a modulatory role in endogenous signalling, despite the fact that it has been critical for therapeutic targeting of SMO. However, there is some evidence that a different site within the TMD may regulate SMO activity in response to Hh ligands. The constitutive signalling activity of SMO-ΔCRD can be suppressed by the transient co-expression of PTC1, suggesting that, at least when over-expressed, PTC1 can inhibit SMO by a mechanism that does not require the CRD [29]. Another hint that a third, undiscovered site exists also comes from the observation that the SMO antagonist itraconazole does not compete for binding with either CRD or TMD ligands [38].

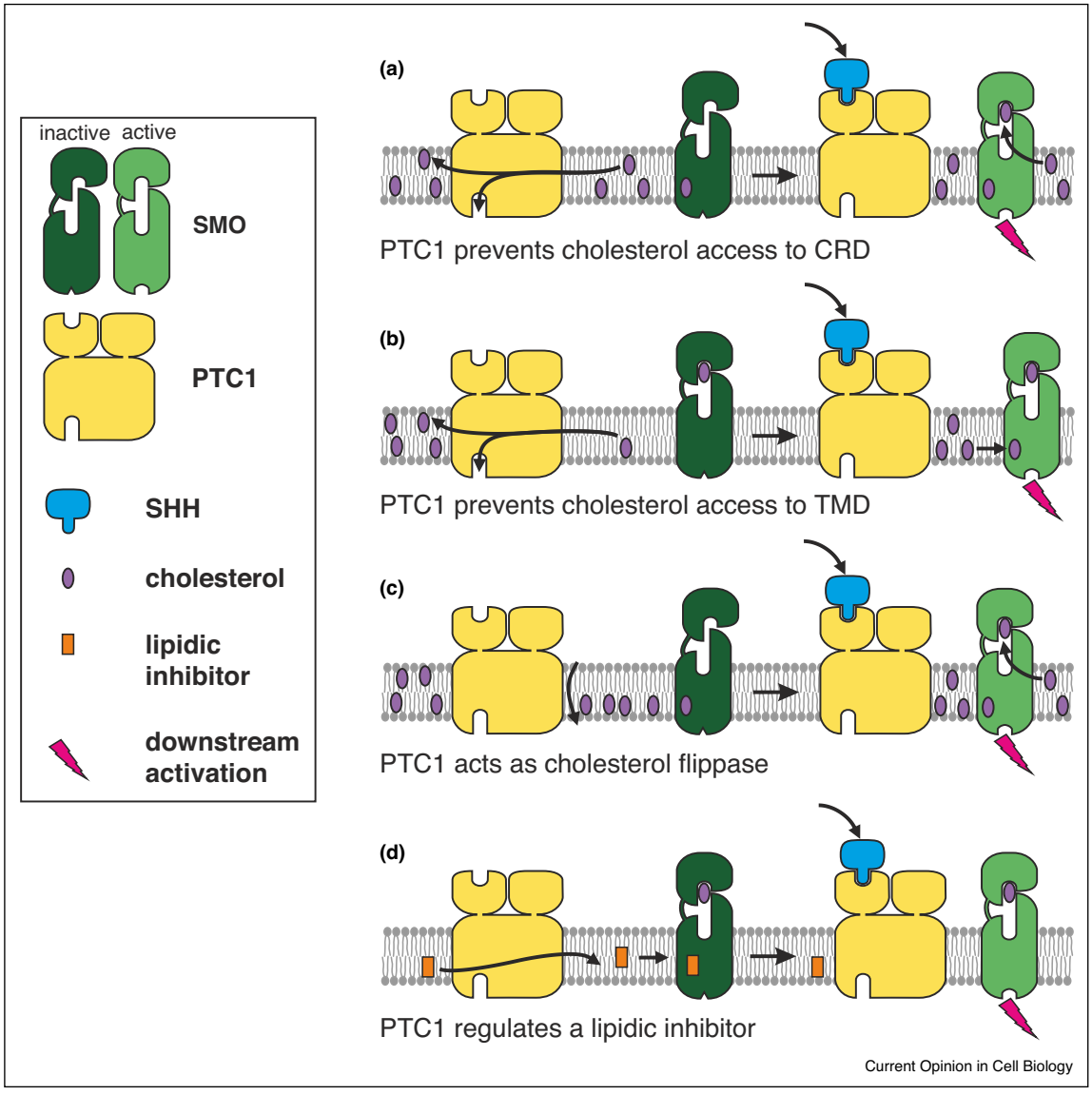
Cholesterol as an endogenous regulator of SMO signalling

Cholesterol, an abundant component of vertebrate cell membranes, is both *necessary* and *sufficient* to activate SMO signalling (summarized in [32••]). A permissive function for cholesterol was first suggested by the observation that cellular cholesterol depletion or drugs that impair intracellular cholesterol transport reduce Hh signalling [39–42]). In addition, humans with mutations in genes encoding enzymes of distal cholesterol biosynthesis, such as Smith-Lemli-Opitz syndrome (SLOS), have Hh-related developmental defects [43,44]. There is uncertainty about whether impaired signalling in SLOS is due to a cholesterol deficit or due to accumulation of an inhibitory precursor derived from 7-dehydrocholesterol (7-DHC), the substrate for the enzyme mutated in SLOS, 7-dehydrocholesterol reductase (7-DHCR) [45]. The cholesterol deficiency model is more likely because signalling can be rescued by cholesterol addition in cells carrying 7-DHCR disease mutants [46•] and defects in enzymes earlier in the cholesterol biosynthesis cascade, which do not lead to elevated 7-DHC, also impair Hh signalling in mice [47].

The permissive role of cholesterol has been attributed to the TMD rather than the CRD, because the constitutive signalling activity of SMO-ΔCRD can be reduced by cholesterol depletion [29], and because a SMO protein carrying a mutation in the CRD sterol-binding site remains sensitive to 7-DHCR inhibitors [46•]. Indeed, many transmembrane receptors, including GPCRs (reviewed in [48]), require interactions with membrane cholesterol around their TM domains for proper function.

While cholesterol is required for signalling by many receptors, the role of cholesterol in SMO signalling is distinguished by the fact that it is *sufficient* to activate the signalling cascade in the absence of native Hh ligands [31••,32••]. This effect, which suggests an instructive rather than a purely permissive role, is mediated by the structurally-defined binding site in the CRD, located nearly ~12 Å away from the lipid bilayer (Figure 3a and b). While exogenous cholesterol activates SMO through the CRD, definitive evidence that endogenous cellular cholesterol (rather than a different lipidic ligand) engages the SMO CRD site in cells is still incomplete. A plausible alternative is that oxysterols are the physiologically-relevant CRD ligands — exogenous cholesterol activates SMO after it is metabolized to oxysterols. Two observations have pointed to cholesterol as the endogenous CRD ligand. First, fluorinated cholesterol derivatives that cannot be metabolized to oxysterols can

Figure 4



Models for PTC1 function. **(a)** In the first model, the SMO TMD is constitutively associated with cholesterol but PTC1 prevents the SMO CRD from accessing cholesterol, thereby preventing activation. Upon SHH-binding, PTC1 is inactivated, allowing the CRD to acquire cholesterol and to become activated. **(b)** In the second model, the SMO CRD contains cholesterol as a necessary co-factor and PTC1 prevents the SMO TMD from accessing cholesterol, thereby preventing activation. Upon SHH-binding, PTC1 is inactivated, allowing the SMO TMD to acquire cholesterol and become activated. **(c)** In the third model, a variant of the first, PTC1 acts as a cholesterol flippase, shifting cholesterol from the outer to the inner leaflet of the lipid bilayer and thereby preventing the SMO CRD from accessing cholesterol. These models are not mutually exclusive and PTC1 could regulate cholesterol access to both sites. **(d)** Cholesterol acts as a co-factor for SMO activation, while PTC1 regulates a different lipidic ligand (solid square) which could either function as a SMO antagonist as shown here or as a SMO agonist.

still activate signalling [31••]. Second, mutations in the sterol-binding groove that impair activation by oxysterols, but not by cholesterol, have little effect on SHH-driven signalling (while mutations that block cholesterol-induced activation also impair SHH-induced activation) [31••,32••].

Taken together, the above evidence has nominated cholesterol as an endogenous, physiologically-relevant regulator of SMO function and led to the hypothesis that PTC1 regulates SMO by preventing its access to cholesterol [31••,32••]. Indeed, SHH and cholesterol can activate Hh signalling in a synergistic fashion [31••,32••]. This model is also consistent with the observation that PTC1 has homology to the Niemann–Pick C1 (NPC1) cholesterol transporter in lysosomes [49] and has been shown to be capable of both binding and transporting cholesterol [50]. The additional homology of PTC1 to RND-family transporters suggests that it may be able to use transmembrane ion gradients to actively transport cholesterol, even against a concentration gradient [7].

An unresolved question is whether PTC1 regulates cholesterol access to the CRD, the TMD, or both. Covalent labeling of the CRD ligand-binding site by cholesterol can be reduced by PTC1 in a SHH-regulated fashion, consistent with the idea that PTC1 can regulate cholesterol access to the CRD [33••]. As mentioned above, mutations in the CRD that block cholesterol binding also reduce SMO signalling in both cells and animals. The case that PTC1 may also regulate access of the TMD to cholesterol is based on the observation that PTC1 overexpression can inhibit the high constitutive activity of SMO-ΔCRD [29].

How might PTC1 prevent SMO access to cholesterol, especially given that cholesterol is such an abundant lipid? Biochemically, PTC1 could change the levels of accessible (or chemically active) cholesterol, which is known to regulate its interaction with proteins and is distinct from the tightly-bound pool that plays a structural role in lipid bilayers [51] (Figure 4a and b). Alternatively, PTC1 could function as a lipid flippase that changes the distribution of cholesterol between the two leaflets of the plasma membrane, an activity analogous to the inferred ability of NPC1 to flip cholesterol from the luminal to the cytoplasmic leaflet of the lysosomal membrane (Figure 4c).

An important caveat to the above discussion is that changes in either local or global cholesterol levels or cholesterol distribution in response to Hh signalling have not yet been demonstrated in an endogenous signalling context. Thus, we cannot rule out the possibility that cholesterol functions as a constitutive co-factor or allosteric regulator of SMO activity, required for SMO to adopt a fully active conformation. In this scenario PTC1 would inhibit SMO through a different small molecule regulator (Figure 4d).

Conclusions and perspectives

The crucial processes of embryonic development and regenerative responses depend upon proper functioning of the Hh signalling pathway. Recent multi-domain structures of SMO have cast a new light on the role of the extracellular domains of SMO and the allosteric interaction between its two defined ligand-binding sites. Functional studies have nominated cholesterol as an endogenous instructive modulator of SMO, mediating the critical regulatory interaction between SMO and the receptor for Hh ligands, PTC1. These insights have advanced our understanding of this key developmental signalling system, suggested strategies to overcome clinically-significant resistance to anti-SMO drugs, and, more generally, suggested the possibility that cholesterol may be used more broadly as a lipid second messenger in signalling systems.

Several important questions remain for future research. Further structural studies will be required to understand how the SMO TMD adopts an active conformation and how it subsequently communicates with candidate downstream regulators, such as GPR161 [52] or heterotrimeric G-proteins [53,54], to eventually influence GLI activity. Understanding the mechanism by which cholesterol gains access to the CRD site, perched ~12 Å above the membrane, will also require further studies, since a cholesterol molecule would have to completely desorb from the membrane to gain access to this site. Finally, since the regulation of SMO by PTC1 in vertebrates is thought to be orchestrated at primary cilia or associated membranes, it will be important to test whether changes in sterol lipids or other endogenous SMO regulators are compartmentalized to cilia-associated membrane compartments.

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