# Multiple roles for lipids in the Hedgehog signalling pathway

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Abstract | The identification of endogenous sterol derivatives that modulate the Hedgehog (Hh) signalling pathway has begun to suggest testable hypotheses for the cellular biological functions of Patched, and for the lipoprotein association of Hh. Progress in the field of intracellular sterol trafficking has emphasized how tightly the distribution of intracellular sterol is controlled, and suggests that the synthesis of sterol derivatives can be influenced by specific sterol-delivery pathways. The combination of this field with Hh studies will rapidly give us a more sophisticated understanding of both the Hh signal-transduction pathway and the cell biology of sterol metabolism.

Inteins
(Protein introns). Enzymatically active domains that splice themselves out of the precursor protein, ligating the protein fragments (the 'exteins') on either side. Inteins can also ligate exteins *in trans* as well as *in cis*, and this has been exploited to modify proteins *in vitro*.

Raft-lipid microdomains
Small phase-separated regions of the cell membranes that are rich in cholesterol and sphingolipids. Their affinity for specific transmembrane and lipid-linked proteins, and their ability to cluster to form higher order structures have been proposed to be important for the regulation of signalling and membrane trafficking.

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The Hedgehog (Hh) signal transduction pathway (BOX 1) has conserved functions throughout metazoan development, controlling patterning, growth and cell migration. It is also expressed widely in adults, regulating tissue homeostasis in the gastrointestinal tract[1], nervous system[2], skin and blood[3]. Misregulation of Hh signalling is at the root of several developmental defects[4,5], and can lead to tumorigenesis in adults[6–9]. Over the past 12 years, accumulating evidence suggests that lipids might regulate this important signal-transduction pathway at many levels. However, the identity of these lipids, their exact function and the logic of their use remain mysterious.

The first clue that lipids might have a special function in Hh signalling came from the identification of a unique processing mechanism that results in the covalent linkage of Hh to cholesterol. Hh is synthesized as a 45 kDa pro-protein that undergoes autocatalytic cleavage by an intein-like mechanism. The C-terminal domain of Hh, which resembles a self-splicing intein domain, catalyses its own removal and replacement by cholesterol. This cleavage results in a 19 kDa N-terminal fragment that is covalently linked at its C terminus to cholesterol[10,11]. The Hh N-terminal fragment is also palmitoylated on a Cys residue near its N terminus[12] by the endoplasmic reticulum (ER) transmembrane protein skinny hedgehog[13–16] (SKI, also known as sightless, rasp or central missing; FIG. 1a). Both lipid modifications are required for full signalling activity *in vivo*[12,13,17–20]. Lipid-modified Hh has a high affinity for cell membranes. Indeed, it is targeted to raft-lipid microdomains in both vertebrate and invertebrate cells[21–23]. The mechanisms that allow the secretion, release and spread of such a molecule have been the subject of intense interest.

This review considers the historical findings that hint at important roles for lipids in Hh trafficking and signalling, and will discuss at length work that has begun to outline the specific functions of lipoproteins and signalling cholesterol derivatives in regulating this important pathway.

## Secretion and trafficking of Hh

***Hh secretion.*** Dispatched is required for Hh secretion[24]. Dispatched contains 12 transmembrane domains and is related to the resistance-nodulation division (RND) family of bacterial proton-driven pumps[25]. Bacterial proteins of the RND family use a proton gradient to transport multiple small lipophilic molecules across the membrane bilayer[26]. The two other metazoan members of this family include the Hh receptor Patched, and the protein encoded by the Niemann–Pick type C1 (*NPC1*) disease gene, which promotes cholesterol efflux from late endosomes. The functions of both Patched and NPC1 will be discussed later in this review. Members of the RND family, including Patched, Dispatched and NPC1, contain two related copies of a signature domain with six transmembrane-spanning regions. Mutations in Dispatched which disturb conserved residues that are important for the function of bacterial transporters, also prevent Hh release[25], consistent with the hypothesis that Dispatched can transport a small molecule across the bilayer.

A fragment of the signature RND domain, called a sterol-sensing domain, is also shared with other proteins that are involved in sterol metabolism. The sterol-sensing domain of HMGCoA reductase (the rate-limiting enzyme in cholesterol biosynthesis) regulates its stability in



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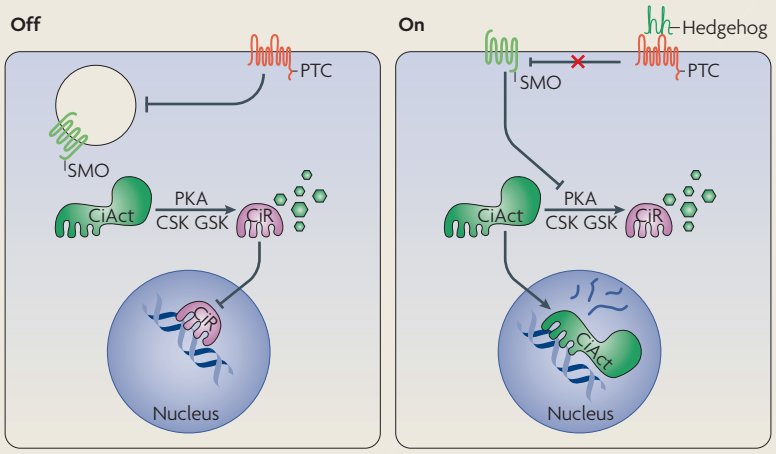
Box 1 | **The Hedghog signalling pathway**




Hedgehog (Hh) signals by binding to Patched (PTC), a protein with 12 transmembrane domains that, as with Dispatched, is related to the resistance-modulation division family of bacterial proton-driven transporters. In the absence of Hh (see figure, left panel), Patched represses the activity of the G-protein-coupled receptor Smoothened (SMO). Repression is associated with reduced Smoothened stability and depletion of the protein from either the plasma membrane (in *Drosophila melanogaster*) or from the primary cilium (in vertebrates)[66,102–104]. In the absence of Smoothened signalling, GLI family transcription factors (Cubitus interruptus (Ci) in *D. melanogaster*) are multiply phosphorylated by protein kinase A (PKA), glycogen synthase kinase (GSK) and casein kinase (CSK). Phosphorylation targets GLI (Ci) for processing by the proteasome, converting the full-length transcriptional activator (CiAct) to a shorter transcriptional repressor (CiR). When Patched-mediated repression is relieved by Hh binding (see figure, right panel), Smoothened moves to the primary cilium or to the plasma membrane. In *D. melanogaster*, it has been shown that Smoothened becomes multiply phosphorylated[105,106], which changes the conformation of its cytoplasmic tail and promotes dimerization[107]. Activated Smoothened then inhibits phosphorylation of GLI (Ci) proteins, thereby preventing degradation of the repressor form and allowing nuclear translocation[108–110].

response to cholesterol. The sterol-sensing domain of SCAP (sterol-regulatory-element-binding protein (SREBP)) cleavage-activating protein) responds to cholesterol levels by altering membrane trafficking and the cleavage of the membrane-associated transcription factor SREBP, which regulates the transcription of genes that are involved in sterol metabolism[27]. Whether Dispatched itself responds to sterol levels is not known, and its precise function in Hh release has not yet been determined.

***Hh spreading.*** How can a lipid-modified protein spread so widely through tissue? Signalling-competent Hh can be isolated from tissue-culture-cell supernatants in high-molecular-weight multimer complexes between 158 and 4,000 kDa (from 6 to at least 160 times the monomer size). Monomer-sized complexes do not signal as efficiently. The formation of the highest molecular-weight complexes depends on both palmitoylation of the N terminus and cholesterol modification of the C terminus (FIG. 1b). Whether these complexes contain other proteins besides Hh is unknown[18,19,23,28,29].

In *Drosophila melanogaster* larvae, lipoprotein particles might help mobilize Hh. Biochemical fractionation of imaginal discs from *D. melanogaster* larvae shows that, although most lipid-modified Hh will form pellets with cell membranes, Hh molecules that remain in the supernatant are almost entirely associated with lipoprotein particles[30]. It will be interesting to determine whether the cholesterol-dependent Hh multimers that are secreted by tissue-culture cells might reflect the association of Hh with serum-derived lipoproteins, or whether multimer formation is a completely distinct mechanism for Hh release.

Lipoproteins comprise a phospholipid monolayer that surrounds a core of esterified cholesterol and triglyceride (FIG. 1c). Insect lipoproteins, called lipophorins, are scaffolded by Apolipophorin[31–33] — a protein that is evolutionarily related to the vertebrate apolipoprotein B (REF. 34). Lipid modifications, such as the addition of cholesterol, palmitate and glycosyl phosphatidylinositol (GPI), that target proteins to the exoplasmic face of the plasma membrane should fit equally well into the outer phospholipid monolayer of lipoproteins (FIG. 1c). Indeed, *D. melanogaster* lipophorin particles also bind to the morphogen molecule Wingless, which is palmitoylated twice, and to several GPI-linked proteins[30,35].

RNA interference-mediated knockdown of lipophorin restricts the range of Hh signalling in *D. melanogaster* imaginal discs: it reduces the activation of long-range target genes but leaves short-range target-gene activation unaffected. In lipophorin-deficient discs, Hh protein is released but accumulates to an abnormally high level in short-range cells[30]. Thus, the role of lipophorin in Hh release is not clear. It is possible that incomplete knockdown of lipophorin produces only an intermediate phenotype. Alternatively, two mechanisms for Hh release might operate in wing discs: a long-range mechanism that depends on lipophorin and a short-range mechanism that is lipophorin-independent. Whether any of the mammalian Hh proteins bind to low-density lipoprotein (LDL) or high-density lipoprotein (HDL)-type particles is unknown, although this would be interesting to investigate.

Cholesterol modification clearly has an important influence on Hh trafficking. The 19 kDa N-terminal Hh domain can be artificially generated in the absence of cholesterol modification by the simple expedient of stop codon insertion or C-terminal domain deletions[11]. This altered protein, termed HhN, is secreted in a Dispatched-independent manner[24], does not form multimeric complexes[18,19,23,28,29], and is distributed differently in both producing and receiving cells[17,18,29]. Although HhN has been reported to spread further, it does not seem to signal as efficiently as cholesterol-modified Hh[10,18,24,29,36]. The increased range of spreading might suggest that the normal function of Hh cholesterol modification is to promote the interaction of Hh with the cell membrane — this interaction could prevent dissociation from receiving tissue and delay Hh movement. However, this simple explanation seems unlikely because the anchors probably interact, either with each other (when Hh is organized as micellar multimers) or with the outer phospholipid monolayer of a lipoprotein (FIG. 1).



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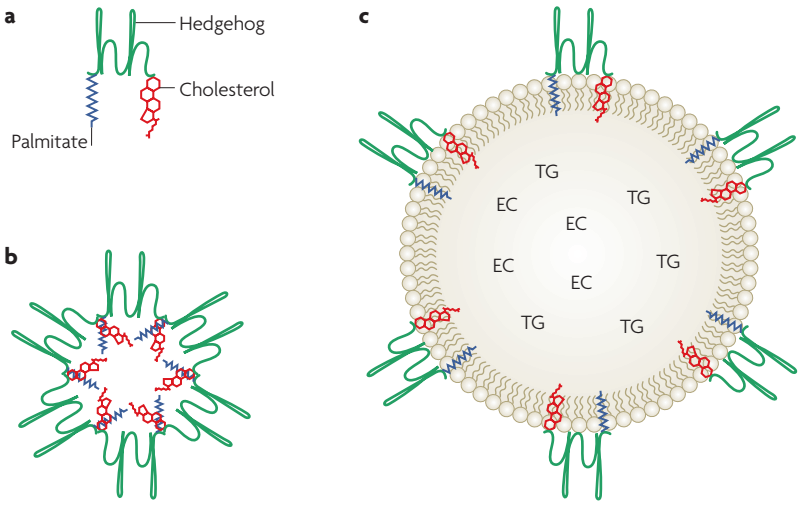


Figure 1 | **Proposed vehicles for Hedgehog release. a** | Hedgehog (Hh; green) is covalently linked to cholesterol (red) and palmitate (blue). **b** | Interaction of the lipid moieties (such as cholesterol and palmitate) with each other drives the formation of Hh multimers. **c** | Lipoproteins consist of an outer phospholipid monolayer (beige) that surrounds a core of esterified cholesterol (EC) and triglyceride (TG). Hh binds to lipoprotein particles through the insertion of lipid moieties (cholesterol and palmitate) into the outer phospholipid monolayer.

Interaction with heparan sulphate proteoglycans (HSPGs) provides a likely explanation for the continuing association of Hh micelles or Hh–lipoprotein complexes with tissue. Lipid-modified Hh does not enter tissue that cannot synthesize heparan sulphate[37–39]. Recent work suggests that lipoproteins interact physically with HSPGs in *D. melanogaster* wing discs[35]. Hh that has interacted with lipoproteins through lipid anchors might therefore be restricted to tissue through these lipoprotein–heparan sulphate interactions. This would be consistent with the observation that only lipid-modified Hh is dependent on HSPGs in order to associate with tissue. Direct binding of Hh to HSPGs might also provide tissue affinity. In this case, Hh multimerization might also promote HSPG binding by increasing the local concentration of heparan sulphate-binding sites on Hh.

## The role of cholesterol in Hh signalling

As illustrated in BOX 1, Patched-mediated repression of Smoothened signalling is essential to keep the Hh pathway inactive in the absence of Hh ligand. Smoothened repression in both *D. melanogaster* and in mammals correlates with changes in its subcellular localization. In flies, the stability and phosphorylation of Smoothened are also altered by Patched-mediated repression. However, the mechanism by which Patched alters Smoothened trafficking and phosphorylation is not understood in detail. It is unlikely that Patched directly binds to Smoothened; sub-stoichiometric amounts of Patched are sufficient to repress Smoothened function in tissue-culture cells[40].

Mammalian Smoothened signalling activity can be regulated by binding to exogenous small lipophilic molecules that are structurally related to sterols. Cyclopamine, a steroidal alkaloid derived from the wild California grass *Veratrum californicum* (FIG. 2), binds to and inactivates mammalian Smoothened[41–43]. By contrast, various small molecule agonists compete with cyclopamine to bind to Smoothened and activate signalling[44,45]. These findings have led to the hypothesis that a structurally related endogenous ligand for Smoothened might exist, and that Patched might regulate its availability. However, none of these exogenous agonists or antagonists has been shown to affect the activity of *D. melanogaster* Smoothened. If there are lipophilic ligands for *D. melanogaster* Smoothened, these might differ from their mammalian counterparts.

The repressive activity of Patched is dependent on the conserved residues that it shares with RND transporters[46]. These transporters use a proton gradient to flux small lipophilic molecules across the bilayer[26,47]. The protein most closely related to Patched is encoded by the *NPC1* gene, another member of the RND transporter family. NPC1 is required for the mobilization of LDL cholesterol from late endosomes, and can act as a proton-driven pump when expressed in bacteria. Based on these analogies, a plausible hypothesis is that Patched regulates the availability of a Smoothened ligand (possibly sterol-related) by pumping it across the bilayer.

***Insights from disease models.*** The importance of sterols in regulating Smoothened signalling has also been suggested by developmental defects that have been observed in diseases of distal cholesterol biosynthesis[4,48,49]. Smith–Lemli–Opitz syndrome (SLO) results from mutations in 7-dehydrocholesterol (7-DHC) reductase. Patients with SLO accumulate the immediate cholesterol precursor 7-DHC (FIG. 2) and have reduced levels of cholesterol. Fibroblasts that have been isolated from patients with SLO exhibit reduced Hh-pathway activity when deprived of exogenous LDL cholesterol[4]. In principle, this might result from either reduced cholesterol or from a negative effector that is derived from 7-DHC, or both. However, lowered cholesterol availability is likely to be at least in part responsible. Removal of LDL cholesterol and the loss of endogenous synthesis of cholesterol from wild-type fibroblasts also inhibits Smoothened activity[4]. Surprisingly, knocking down 7-DHC reductase in *Xenopus* activates rather than inhibits Hh signalling[50].

Recent studies of mouse mutants for both INSIG1 (insulin-induced gene-1) and INSIG2 have suggested that the accumulation of cholesterol precursors might also exert negative effects on the Hh pathway[51]. INSIG1 and INSIG2 proteins are transmembrane proteins of the ER that restrict both the biosynthesis and uptake of cholesterol when cholesterol is abundant. Removing both INSIG1 and INSIG2 increases cholesterol biosynthesis, but also elevates the levels of cholesterol precursors such as 7-DHC. Although these mice resemble SLO mice in the elevation of 7-DHC (REF. 52), they have increased rather than decreased levels of cholesterol[51]. INSIG1 INSIG2 double-knockout mice share some of the phenotypic abnormalities of SLO, including a cleft palate[4,51].

Cleft palate
A craniofacial abnormality that results from failure to fuse the left and right palatal shelves at the midline during embryogenesis. It can be caused by several environmental and genetic factors, including defects in Sonic Hh signalling.



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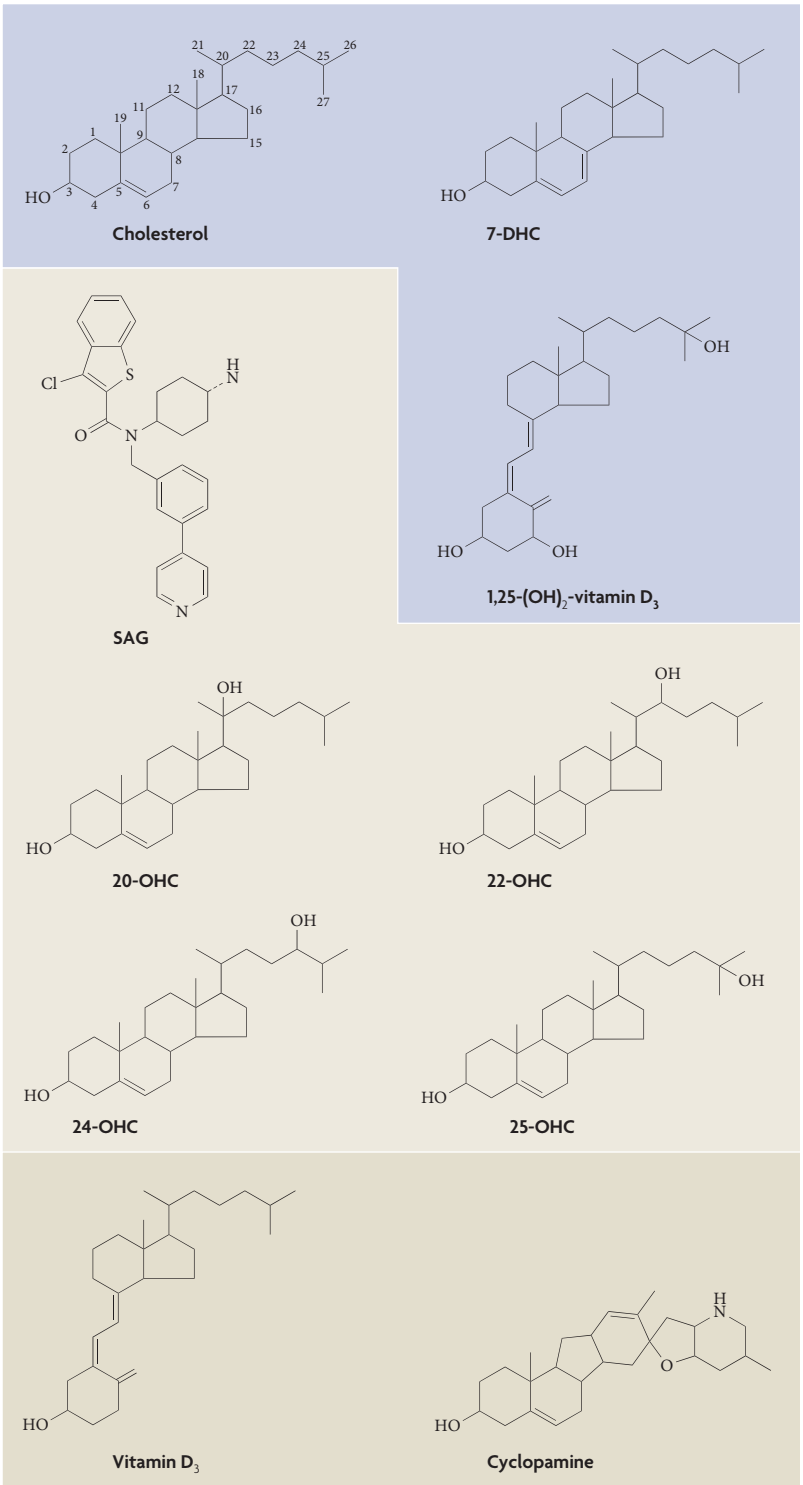


Figure 2 | **The structures of sterol-related molecules.** The structure of cholesterol is shown (top left), with the numbering system used to indicate the different carbon atoms. The enzyme that converts the immediate cholesterol precursor 7-dehydrocholesterol (7-DHC; top right) to cholesterol is missing in patients with Smith–Lemli–Opitz syndrome. The molecules that stimulate Hedgehog (Hh) signalling include oxysterol derivatives of cholesterol and the Smoothened agonist SAG, a synthetic small molecule. The structures of hydroxylated sterols (20-hydroxycholesterol (20-OHC); 22-OHC; 24-OHC; 25-OHC) and 1,25-$(OH)_2$-$D_3$ are shown. Molecules that inhibit Hh signalling include the natural steroid hormone vitamin $D_3$ and the plant steroidal alkaloid cyclopamine. Whether hydroxylation at $C_1$ and $C_{25}$ influences the activity of vitamin $D_3$ with respect to Hh signalling is unknown.

This seems to indicate that the build-up of precursors, rather than reduced cholesterol levels, can cause some of the defects seen in SLO. It will be interesting to specifically examine whether the Hh signalling pathway is altered in these mice.

***Vitamin $D_3$ negatively regulates Hh signalling.*** Recently, the identification of specific endogenous cholesterol derivatives that influence mammalian Smoothened activity has confirmed that the pathway can be both positively and negatively influenced by signalling sterols, perhaps at different steps. Work from the Pepplenbosch laboratory has identified an endogenous sterol derivative, vitamin $D_3$ (FIG. 2), that seems to repress Smoothened signalling activity when applied to C3H/10T1/2 fibroblasts or to developing zebrafish embryos[53]. Transfection of Smoothened increased vitamin $D_3$ binding to yeast cells, which suggests that vitamin $D_3$ might bind to Smoothened. This study also identified a Smoothened inhibitor of moderate activity in C3H/10T1/2 fibroblast supernatants in which Patched expression has been increased by transfection. Production of this inhibitor is reduced by pravastatin and rescued by mevalonate, which indicates that it could be a sterol derivative. However, *in vivo,* vitamin $D_3$ must be derived from nutritional sources or by UV irradiation of 7-DHC in the skin[54]. Nutritionally derived vitamin $D_3$ is carried in lipoproteins, whereas the product of *de novo* synthesis in the skin travels through the circulation in complexes with vitamin-D-binding protein (VDBP)[55]. Thus, if vitamin $D_3$ is an endogenous Smoothened repressor, then it cannot be produced *de novo* from cells that produce Patched. The fact that endogenous sterol synthesis is required for the release of the Patched-dependent Smoothened inhibitor in culture precludes vitamin $D_3$ as the Smoothened inhibitor, but points to another related molecule produced by these cells.

The twice-hydroxylated derivative of Vitamin $D_3$, 1,25-$(OH)_2$-$D_3$ (FIG. 2), is a steroid hormone with important functions in maintaining calcium homeostasis, and in the control of cell differentiation and proliferation in many tissues[56]. It is not clear how hydroxylation affects the activity of vitamin $D_3$ with respect to the Hh pathway. Nevertheless, it is interesting to note that 1,25-$(OH)_2$-$D_3$ inhibits the growth of many tumours in which the Hh pathway is active[57–60]. Furthermore, 7-DHC — the immediate precursor of vitamin $D_3$ — is the cholesterol precursor that accumulates to high levels in SLO syndrome. However, it remains controversial whether elevated 7-DHC levels, as opposed to reduced cholesterol levels, cause the defects seen in SLO. Reduced cholesterol could inhibit the production of positively acting cholesterol derivatives, as described in the next section.

***Oxysterols positively regulate Hh signalling.*** Cholesterol can be hydroxylated at different positions by enzymes located in the ER and in the mitochondria (FIG. 2). In contrast to vitamin $D_3$, hydroxylated cholesterol derivatives positively influence Hh signalling in two separate systems:



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mesenchymal stem cells and medulloblastoma (MB) cell lines. The combination of 20- and 22-hydroxycholesterol (20-OHC and 22-OHC; see FIG. 2) promotes the differentiation of bone and inhibits the differentiation of adipose tissue from mesenchymal stem cells[61]. Recent work from the Beachy and Parhami laboratories has shown that osteogenic differentiation in response to oxysterols requires Smoothened signalling and that the addition of 20-OHC and 22-OHC, but not 7-OHC (cholesterol), increases the activation of Hh target genes[62].

Work from the Scott laboratory focused on the role of oxysterols in Hh signalling in the MB cell line PZp$^{53}$Med (REF. 63). These cells do not express Patched and therefore constitutively activate Hh target genes and proliferation[64]. Blocking distal cholesterol biosynthesis with triparanol, an inhibitor of 3β-hydroxysterol-Δ24 reductase, inhibits both proliferation and transcription of a Hh signalling reporter construct in PZp$^{53}$Med cells. Signal transduction and proliferation are restored by adding cholesterol, but addition of specific oxysterols is at least tenfold more potent. 20-OHC and 22-OHC are effective at activating Hh reporters, 24-OHC and 25-OHC also have significant activity, whereas 7-OHC does not affect the activation of Hh reporters. Oxysterols increase Hh signalling in MB cells and mesenchymal stem cells.

How do oxysterols function to stimulate Hh signalling? Oxysterols, unlike the Smoothened agonist SAG (FIG. 2), do not compete with cyclopamine to bind to Smoothened. Cyclopamine addition is sufficient to block oxysterol-mediated activation[62]. Oxysterols are unlikely to displace a negative regulatory ligand from Smoothened, but rather function upstream of this step. One possibility might be that oxysterols inhibit Patched activity. However, if oxysterols acted only by inhibiting Patched then they should not further increase constitutive signalling in Patched-deficient cells. This depends on the cell type or experimental conditions. Although addition of oxysterol does not increase Hh reporter activity in *Ptc1*$^{-/-}$-mouse embryonic fibroblasts[62], it does result in a threefold increase in Hh signalling in PZp$^{53}$Med cells[63], which do not express Patched[64].

It is interesting to note that these experiments were done using different amounts of added serum (0.5% in REF. 64 compared with 5% in REF. 62). Serum is a source of lipoproteins, and lipoprotein internalization increases oxysterol synthesis[65]. It is possible that different basal rates of endogenous oxysterol production determine whether the addition of exogenous oxysterols further stimulates Hh signalling in the absence of Patched. It seems that oxysterols probably do not control Smoothened activity by inhibiting Patched, but rather act at a subsequent step. Consistent with this hypothesis, addition of oxysterols mimics the effects of Patched loss-of-function on Smoothened trafficking without affecting Patched levels or localization[66].

How might oxysterols modulate Smoothened localization? Oxysterols are potent ligands that bind to and regulate different proteins. They bind to the liver X receptor (LXR), a nuclear receptor that regulates the transcription of genes that are involved in cholesterol homeostasis[67]. However, LXR agonists do not activate Hh target genes in mesenchymal stem cells[62], which suggests that oxysterols do not function through LXR to modulate Hh signalling. Oxysterols also bind to a family of conserved oxysterol-binding protein (OSBP)-related proteins (ORPs), some of which regulate membrane trafficking[68,69]. It would be interesting to find out whether any of these proteins might influence Smoothened localization. Oxysterol binding to INSIG promotes the interaction of INSIG with SCAP and retention of the SCAP–SREBP complexes in the ER[70].

Do oxysterols regulate Smoothened trafficking by binding to an analogous Smoothened-interacting protein? Investigating the mechanism of oxysterol action should provide a new entry point to understanding Smoothened trafficking and activation.

## Sterol trafficking and the Hh pathway

The ability of specific sterol derivatives to positively or negatively regulate Smoothened signalling raises the interesting question of how the uptake, synthesis and intracellular distribution of these molecules is controlled and whether Patched might somehow influence these processes. This section discusses how oxysterols and vitamin $D_3$ are produced and distributed (also reviewed in REFS 27,56,71,72) and attempts to relate this information to the Hh pathway.

***Intracellular transport of sterols and their derivatives.*** Cellular cholesterol is derived from endogenous synthesis in the ER and from internalized lipoproteins. Cells actively develop and maintain significantly different cholesterol concentrations in different membrane compartments. Many vesicular and non-vesicular mechanisms exist to actively transport cholesterol between different membrane compartments and regulate its distribution within the cell. The first requirement for the synthesis of oxysterols is the delivery of cholesterol to intracellular sites where relevant enzymes are localized. The enzymes that produce oxysterols have been isolated from the ER and Golgi (cholesterol-25-hydroxylase and cholesterol-24-hydroxylase) and from the mitochondria (cholesterol-27-hydroxylase)[73] (FIG. 3a). The site of 20-OHC and 22-OHC production is unknown and the enzymes have yet to be cloned, although consistent enzymatic activity has been detected in mitochondria from adrenal cells.

Recent data suggest that biosynthesis of some sterol derivatives is dependent on specific intracellular cholesterol trafficking machineries. High-level synthesis of 25-OHC and 27-OHC is dependent on NPC1-dependent cholesterol mobilization[65]. NPC1 activity in late endosomes is necessary to mobilize the cholesterol that is derived from internalized LDL and from the plasma membrane to other cellular compartments. NPC1 expression in bacteria can promote influx of fatty acids and acriflavine, but not cholesterol[74]. Whether cholesterol is the real substrate of NPC1 in vertebrate cells is unclear; it is possible that NPC1 promotes the efflux of cholesterol indirectly, by changing the concentration of other lipids in the endosomes.



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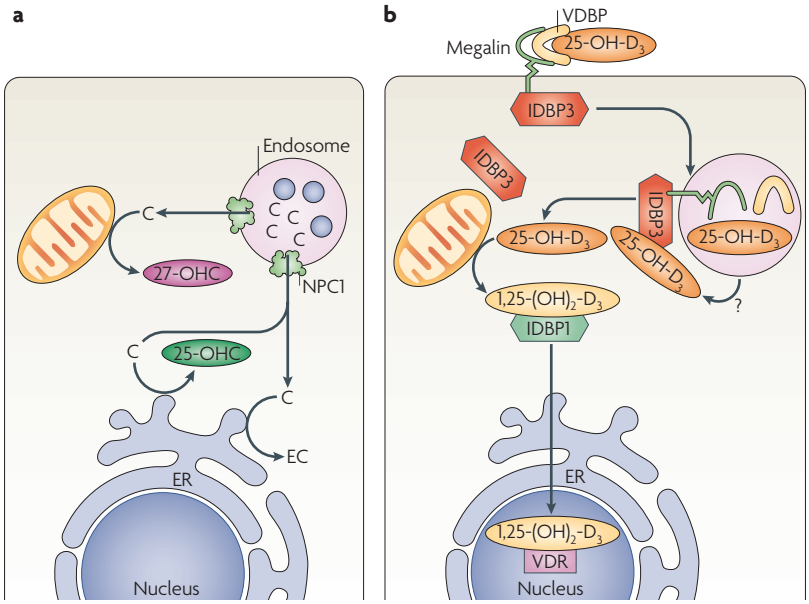


Figure 3 | **Intracellular transport of sterols and sterol derivatives. a** | Niemann–Pick type C1 (NPC1) protein promotes cholesterol (C) trafficking from late endosomes to sites of oxysterol and esterified cholesterol (EC) synthesis on mitochondria and the endoplasmic reticulum (ER). Blue circles depict lipoproteins. **b** | 25-OH-$D_3$ is internalized with vitamin-D-binding protein (VDBP) by Megalin. Binding of intracellular D-binding protein-3 (IDBP3) to 25-OH-$D_3$ promotes transport to mitochondria, where 25-OH-$D_3$ is hydroxylated to 1,25-$(OH)_2$-$D_3$. IDBP1 promotes the delivery of 1,25-$(OH)_2$-$D_3$ to its receptor (VDR) in the nucleus.

The molecules that subsequently deliver endosome-derived cholesterol to different subcellular locations, such as mitochondria, the plasma membrane or the ER, are not completely clear. The steroidogenic acute regulatory protein (START) domain protein MLN64, which binds to cholesterol and is also present on NPC1-containing endosomes, has been suggested to have a role in transferring cholesterol from the endosomal membrane to other membranes[75]. OSBP and other ORPs have also been suggested as candidates for the transport of cholesterol to other membrane compartments[76]. In NPC1-deficient cells, cholesterol accumulates in late endosomes and its delivery to the plasma membrane or mitochondrial and ER compartments is reduced[77–79].

Under normal conditions, cholesterol that is derived from LDL uptake is delivered to sites of oxysterol synthesis in the ER and mitochondria. Oxysterols then function through LXR to reduce cellular cholesterol levels by activating the transcription of genes that are involved in cholesterol efflux[80,81]. NPC1-mutant cells do not synthesize oxysterols in response to LDL uptake, and therefore accumulate abnormally high levels of total cellular cholesterol — this phenotype can be rescued by the addition of oxysterols[65]. Whether a particular NPC1-mutant protein can promote cholesterol trafficking to sites of oxysterol synthesis is uncorrelated with its ability to deliver cholesterol to sites of cholesterol esterification in the ER[65]. This suggests that different NPC1-dependent mechanisms might promote cholesterol trafficking to these different locations (FIG. 3a).

Dauer formation
When starved of nutrients, the nematode worm *C. elegans* progresses to a unique larval form called a dauer. Dauers are long-lived and resistant to environmental insults but do not reproduce. When conditions are favourable, dauer larvae re-enter reproductive development.

NPC1-dependent cholesterol transport to mitochondria is also essential for the generation of a subset of steroid hormones (FIG. 3a). In *D. melanogaster*, NPC1 is required for synthesis of the steroid hormone ecdysone[82,83], which regulates the transition between different developmental stages in this organism. In *Caenorhabditis elegans*, NPC1 homologues are needed for synthesis of sterol-derived hormones that regulate dauer formation[84]. In vertebrates, synthesis of neurosteroids is reduced in NPC1 mutants[85], but the levels of circulating steroid hormones such as testosterone, progesterone and corticosterone are not affected[86].

The steroid hormones corticosterone and aldosterone rely on an NPC1-independent mechanism for the delivery of their cholesterol precursor. The main source of components for the synthesis of these hormones is the selective uptake of cholesterol esters from HDL through the scavenger receptor B1 (SRB1)[87,88]. Cholesterol esters absorbed by the selective uptake pathway are delivered to lipid droplets, and the release of cholesterol for transport to mitochondria depends on de-esterification by hormone-inducible lipase (HLIP)[89–91].

How might Patched and Hh influence the availability of regulatory sterol derivatives? In principle, Patched and Hh might regulate production, intracellular trafficking or turnover of these molecules. The observation that specific lipoprotein-dependent delivery pathways, such as selective uptake, can influence the efficiency with which sterol derivatives are synthesized suggests an intriguing possible role for the association of Hh with lipoproteins: could the presence of Hh on lipoproteins influence the use of sterols or sterol derivatives present in these particles?

The mechanisms that support hydroxylation of 25-OH-$D_3$ provide an illustration of the importance of facilitated delivery of sterol to specific subcellular locations (FIG. 3b). Vitamin $D_3$ itself is a prohormone; the active form that maintains calcium homeostasis, 1,25-$(OH)_2$-$D_3$, is formed by hydroxylation — first at $C_{25}$ in the liver, and subsequently at $C_1$ in various tissues, including the kidney[56]. Hydroxylation of 25-OH-$D_3$ to form 1,25-$(OH)_2$-$D_3$ is enhanced by specific cellular mechanisms that internalize 25-OH-$D_3$ and deliver it to the 1-hydroxylase. Circulating 25-OH-$D_3$–VDBP complexes are internalized by the lipoprotein receptor-related protein Megalin[92]. The cytoplasmic tail of Megalin binds to two proteins with affinity for 25-OH-$D_3$: intracellular D-binding protein-1 (IDBP1) and IDBP3 (REF. 93). VDBP is degraded in endosomes and 25-OH-$D_3$ moves across the endosomal membrane by an unknown mechanism and binds to IDBP3, which promotes delivery to the mitochondria where the 1-hydroxylase is located. IDBP1 increases delivery of 1,25-$(OH)_2$-$D_3$ to the vitamin D receptor (VDR), a nuclear hormone receptor that mediates many of the transcriptional outputs of vitamin $D_3$ signalling[94–96].

***Models for Patched-mediated Smoothened repression.*** Patched could function similarly to NPC1 by promoting the efflux of cholesterol, but it could also function by directing the delivery of cholesterol to different subcellular compartments — inhibiting the production of positive regulators or increasing the synthesis



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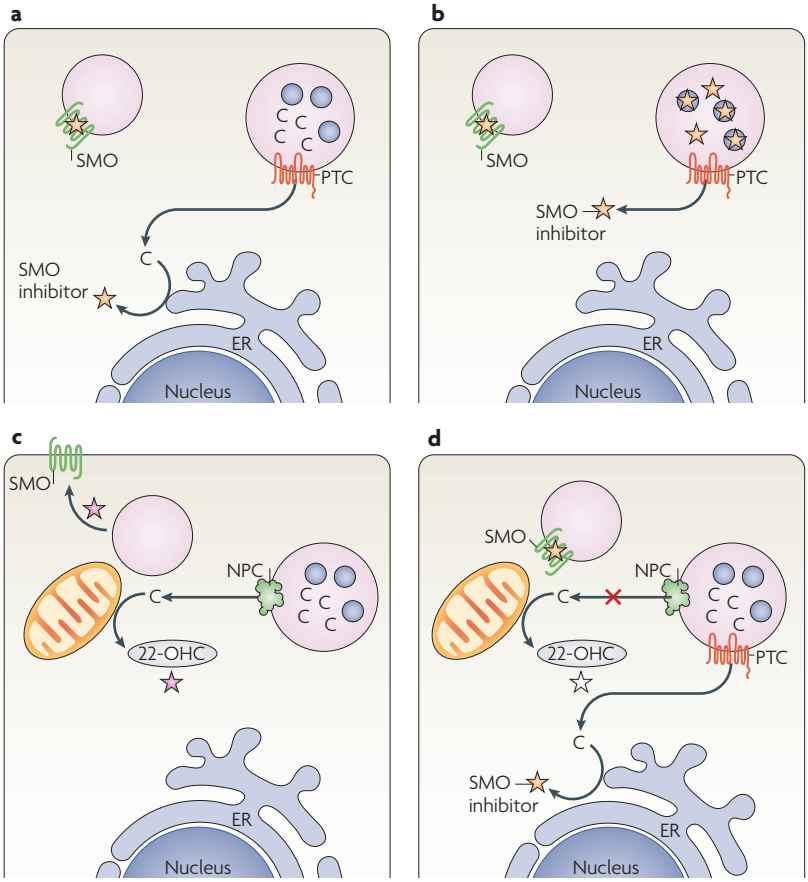


Figure 4 | **Possible models for Patched-mediated Smoothened repression.** **a** | Patched (PTC) acts like other resistance-nodulation division family members as a transporter to mobilize cholesterol (C) across the plasma membrane to allow specific delivery to sites where a Smoothened (SMO) inhibitor (yellow star) can be synthesized from it. Binding of the inhibitor to Smoothened represses its activity. Internalized lipoproteins are shown as blue circles. **b** | A lipophilic compound that inhibits Smoothened is already present in internalized lipoproteins. Patched acts as a transporter and mobilizes this molecule across the membrane and into the cell, making it available to repress Smoothened. **c** | Cellular cholesterol is efficiently transported to sites where Smoothened agonists (purple star) such as 22-hydroxycholesterol (22-OHC) are synthesized, but only in the absence of Patched activity. **d** | When Patched is present, intracellular cholesterol transport is redirected, reducing the synthesis of 22-OHC and/or promoting synthesis of a Smoothened inhibitor. ER, endoplasmic reticulum.

of an inhibitor (FIG. 4). Hh signalling is reduced in tissue-culture cells by drugs, such as progesterone and U18666A, that mimic the NPC1 loss-of-function phenotype and cause cholesterol accumulation in late endosomes[97]. Although NPC1 does not seem to influence Hh signalling in vertebrates, these data suggest an important function for intracellular cholesterol trafficking in regulating Hh signalling. Patched is found in the same late endosomal compartment as its relative NPC1 — indeed, these two proteins co-localize in tissue-culture cells[97]. It would be interesting to compare the accumulation of different sterol metabolites in cells with different levels of Patched activity. An alternative possibility is that Patched might translocate the small lipophilic inhibitor itself (FIG. 4b).

If vitamin $D_3$ is a physiological regulator of Hh signalling *in vivo*, it must be acquired exogenously. Known routes of acquisition include the lipoprotein-mediated delivery of nutritional vitamin $D_3$ and Megalin-mediated internalization of vitamin $D_3$ in complex with VDBP[55]. Hh can be internalized by Megalin[98,99], and the holoprosencephaly observed in Megalin mutants suggests that Hh signalling might be defective[100]. It would be interesting to investigate whether Hh might antagonize uptake of vitamin $D_3$ by Megalin. Although it seems likely that Vitamin $D_3$, as with cholesterol, would require some cellular machinery to promote its efflux from endosomes, no such machinery has yet been identified. It would be interesting to see whether Patched might have such a function (FIG. 4c). Also, in the case of lipoprotein-mediated vitamin $D_3$ delivery, the presence or absence of Hh on lipoproteins might have the capacity to regulate their use.

The levels of intracellular oxysterols will also depend on the rate at which they are metabolized or removed from cells. How does this normally occur? Esterification is one method for the sequestration of oxysterols in lipid droplets. Cells can also actively remove oxysterols through ATP-binding cassette (ABC) transporter-mediated efflux across the plasma membrane[101]. It is currently unclear how or whether the mechanisms that promote metabolism or efflux of oxysterols might be regulated. Hydroxylation of 1,25-$(OH)_2$-$D_3$ at the $C_{24}$ position inactivates this molecule, at least with respect to signalling through the VDR, thereby controlling intracellular levels of the active ligand. Regulation of signalling sterol turnover is another possible control point with the potential to be influenced by the Hh pathway.

## Conclusions and future perspectives

Hh activates its signal transduction pathway by binding to Patched, and preventing Patched from repressing the G-protein-coupled receptor Smoothened. In the absence of Hh, Patched-mediated Smoothened repression occurs by an unknown mechanism that involves alterations in Smoothened trafficking and stability. Recent work has shown that Smoothened signalling activity can be modulated by sterol derivatives. Vitamin $D_3$, and perhaps another related sterol derivative, act negatively to repress Smoothened activity. Specific oxysterol derivatives activate the pathway upstream of Smoothened, possibly by regulating Smoothened trafficking. Patched might therefore function by regulating the availability of such molecules, and its similarity to bacterial proton-driven transporters and to NPC1 suggests that it might promote the transmembrane transport of a small lipophilic molecule. It will be interesting to examine whether Patched has transporter activity, as NPC1 does, and to identify its cargo.

Advances in our knowledge of the uptake and intracellular trafficking of sterols and sterol derivatives provides a framework which can be used to analyse the biological function of Patched and Hh, and also to suggest new avenues for research. Lipophilic molecules that regulate Smoothened activity might be sought in the lipoprotein particles with which Hh associates and is internalized. Investigating the effects of Patched on the trafficking of sterols and their derivatives might also yield important insights into the Hh signalling pathway.

ABC transporter
The many different proteins of the ABC transporter family use ATP to transport small molecules across the plasma membrane. ABCA1 has an important function in reverse cholesterol transport — effluxing cellular cholesterol to HDL particles. ABCG5 and G8 efflux cholesterol and plant sterols out of gut cells and into the gut lumen, regulating dietary sterol uptake.



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## DATABASES

**Interpro:** http://www.ebi.ac.uk/interpro
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apolipoprotein B | 7-DHC reductase | Dispatched | IDBP1 | Hedgehog | INSIG1 | INSIG2 | LXR | Megalin | MLN64 | NPC1 | OSBP | Patched | SCAP | SKI | Smoothened | SRB1 | SREBP

## FURTHER INFORMATION

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