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The role of cholesterol in Shh signaling and teratogen-induced holoprosencephaly

J. P. Incardona and H. Roelink*

Department of Biological Structure and Center for Developmental Biology, University of Washington, Box 357420, HSB G527, Seattle (Washington 98195, USA), Fax + 1 206 543 1524, e-mail: roelink@u.washington.edu

Abstract. Holoprosencephaly, or an undivided forebrain, is a complex brain malformation associated with *Sonic hedgehog* (Shh) mutations. Other causes of holoprosencephaly have converged upon the Shh signaling pathway: genetic and pharmacologic impairment of cholesterol synthesis, and the action of the steroidal alkaloid cyclopamine. This review focuses on recent studies aimed at determining how Shh signaling is affected by these causes of holoprosencephaly, whether they involve a common mechanism and the role played by cholesterol. Cholesterol is potentially important for both biogenesis of Shh and in signal transduction in Shh-responsive cells. Teratogens that induce holoprosencephaly appear to affect Shh signal transduction rather than Shh biogenesis. Analysis of these agents and other compounds that affect various aspects of cellular cholesterol distribution indicates that the role of cholesterol in Shh signal transduction is novel and complicated. The similarity of the Shh receptor, Patched (Ptc), to the Niemann-Pick C1 protein, which is involved in the vesicular trafficking of cholesterol, provides insight into the role of cholesterol and the action of compounds like cyclopamine.

Key words. Sonic hedgehog; patched; Niemann-Pick C1; cholesterol; cyclopamine; signal transduction.

Shh signaling and teratogen-induced holoprosencephaly

The role of Sonic hedgehog in patterning the mammalian forebrain is exemplified by the striking malformations arising from *Shh* mutations. In both mice and humans, *Shh* mutations produce holoprosencephaly, or an undivided forebrain, and associated craniofacial malformations such as cyclopia [1, 2]. Other genetic and environmental causes of holoprosencephaly implicate cholesterol in Shh signaling. Holoprosencephaly is produced in the offspring of pregnant rats treated with AY-9944, an inhibitor of the final step of cholesterol synthesis catalyzed by Δ7-dehydrocholesterol reductase (7DHCR) [3]. Less severe forms of holoprosencephaly are found in a small percentage of patients with Smith-Lemli-Opitz syndrome, a disorder that results from mutations in the gene encoding 7DHCR [4–6]. These conditions result in both low levels of tissue cholesterol and accumulation of 7-DHC and other precursor sterols (fig. 1A) [3–5, 7]. A mild form of holoprosencephaly also resulted in mouse embryos null for *gp330/megalin*, which encodes a member of the low density lipoprotein (LDL) receptor family expressed in neuroepithelium [8].

During the first half of this century, very striking cases of cyclopia and severe holoprosencephaly were common in lambs born on sheep ranches in the Rocky Mountain regions of the western United States. Elegant epidemiological studies and the astuteness of a local shepherd led to the identification of a common range plant as the etiology (see [9] for review). The corn lily, *Veratrum californicum*, was subsequently found to produce two steroidal alkaloids (fig. 1B), cyclopamine and jervine, and a glycoside derivative, cycloposine, which in pure form produced holoprosencephaly when administered to any gastrulation-stage amniote embryo (reviewed in [10, 11]). The steroidal nature of these compounds also suggests that their action could be related to a role for cholesterol in Shh signaling.

* Corresponding author.

Two basic questions arise from these phenomena: what is the role for cholesterol in Shh signaling, and how do these diverse etiologies affect that role? Current research has focused on determining whether holoprosencephaly in these cases is in fact a result of effects on Shh signaling, and if so, whether a common mechanism is involved. As shown schematically in figure 2, common mechanisms that have been suggested include (i) inhibition of the cholesterol-dependent posttranslational modification of Shh; (ii) disruption of cholesterol-rich plasma membrane microdomains ('rafts') potentially involved in Shh signal transduction; and secondary effects on Shh signal transduction as a consequence of either (iii) reduction of cellular cholesterol levels in tissues in which the Shh pathway is active or (iv) disruption of intracellular cholesterol transport in these tissues. However, recent work in our laboratory suggests that a common mechanism cannot be invoked for the effects of teratogenic agents such as AY-9944 and cyclopamine. First, the role of cholesterol in Shh signal transduction appears to be novel and unlike that characterized for other signal transduction pathways. Second, cyclopamine appears to have an effect on Shh signal transduction that is unrelated to cholesterol synthesis or transport. The finding that the Shh receptor, Patched (Ptc), is structurally similar to the Niemann-Pick C1 (NPC1) protein, which is involved in the vesicular trafficking of cholesterol, provides insight into both the role of cholesterol and how cyclopamine may antagonize Shh signal transduction. An analysis of the Ptc/NPC1 relationship also suggests revisions in the current model for Hh signal transduction.

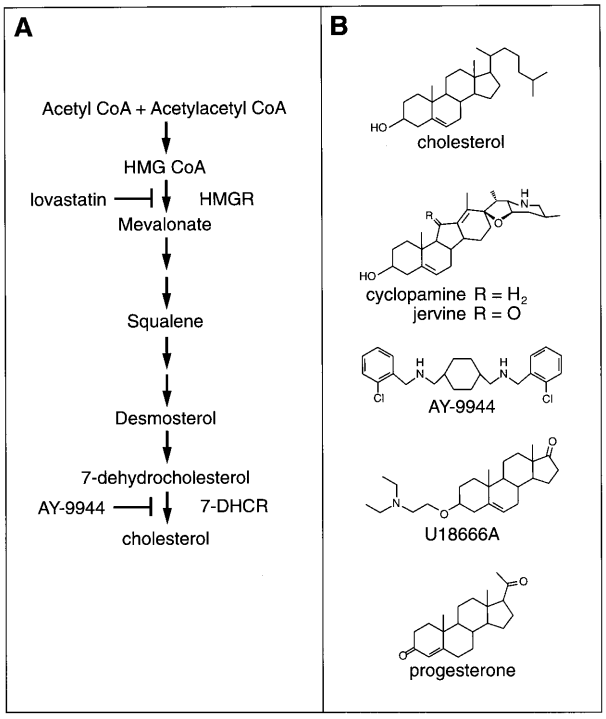

Figure 1. (*A*) Key steps in the cholesterol biosynthetic pathway. The synthesis of mevalonate, a nonsterol intermediate, is catalyzed by hydroxymethylglutaryl-coenzyme A reductase (HMGR). A series of isoprenoid intermediates, synthesized from mevalonate, leads to squalene, which is subsequently cyclized into the first sterol intermediate. A series of demethylations and reductions then leads to 7-dehydrocholesterol (7-DHC). The conversion of 7-DHC to cholesterol is catalyzed by 7-dehydrocholesterol reductase (7DHCR). Inhibition of HMGR by lovastatin results in no sterol accumulation. Inhibition of 7DHCR by AY-9944 results in the accumulation of 7-DHC and other sterol intermediates and byproducts. (*B*) Structures of cholesterol and the primary Shh inhibitors described in the text. Note that cyclopamine and jervine are identical except for the presence of a keto group on C-11 of jervine, which is associated with reduced inhibitory potency in Shh signaling assays, but not in assays of cholesterol transport. Note further that excluding progesterone, all inhibitory compounds are hydrophobic secondary or tertiary amines.

The role of cholesterol in Shh biogenesis and potential effects of holoprosencephaly-inducing teratogens

Hh proteins are synthesized as a 45-kDa precursor which is cleaved autoproteolytically to generate a 19-kDa amino-terminal fragment possessing the signaling activity [12–14]. During cleavage, cholesterol is covalently attached to the N-terminal fragment and anchors mature Hh in the plasma membrane of cells that synthesize the protein [15]. A significant fraction of Shh also is modified on an N-terminal Cys by palmitate [16]. The lipid-modified peptide associates with cholesterol/sphingolipid-rich membrane microdomains ('rafts') ([17] and JPI, unpublished observations) thought to be involved in polarized protein sorting and the spatial organization of signal transduction (see [18] for review). In purified form, lipid-modified Shh is significantly more potent than Shh lacking the lipid modifications [16].

The initial association of holoprosencephaly with reduced 7DHCR activity led to the hypothesis that these conditions result in impaired Shh biogenesis, secondary to reduced cholesterol levels [4] (fig. 2, point 1). However, a number of sterols, including 7-DHC and other precursors, were shown to function as well as cholesterol in an in vitro Shh autocatalytic processing assay [19]. Similarly, it is unlikely that cyclopamine and jervine cause holoprosencephaly at the level of Shh biogenesis, because teratogenic concentrations of these compounds did not affect Shh biogenesis in either COS-1 or HK293 cells [19, 20]. Nevertheless, it has not been determined whether Shh modified by sterols other than cholesterol has the same signaling activity. Furthermore, it is unknown whether attachment to different sterols would affect the association of Shh with rafts,

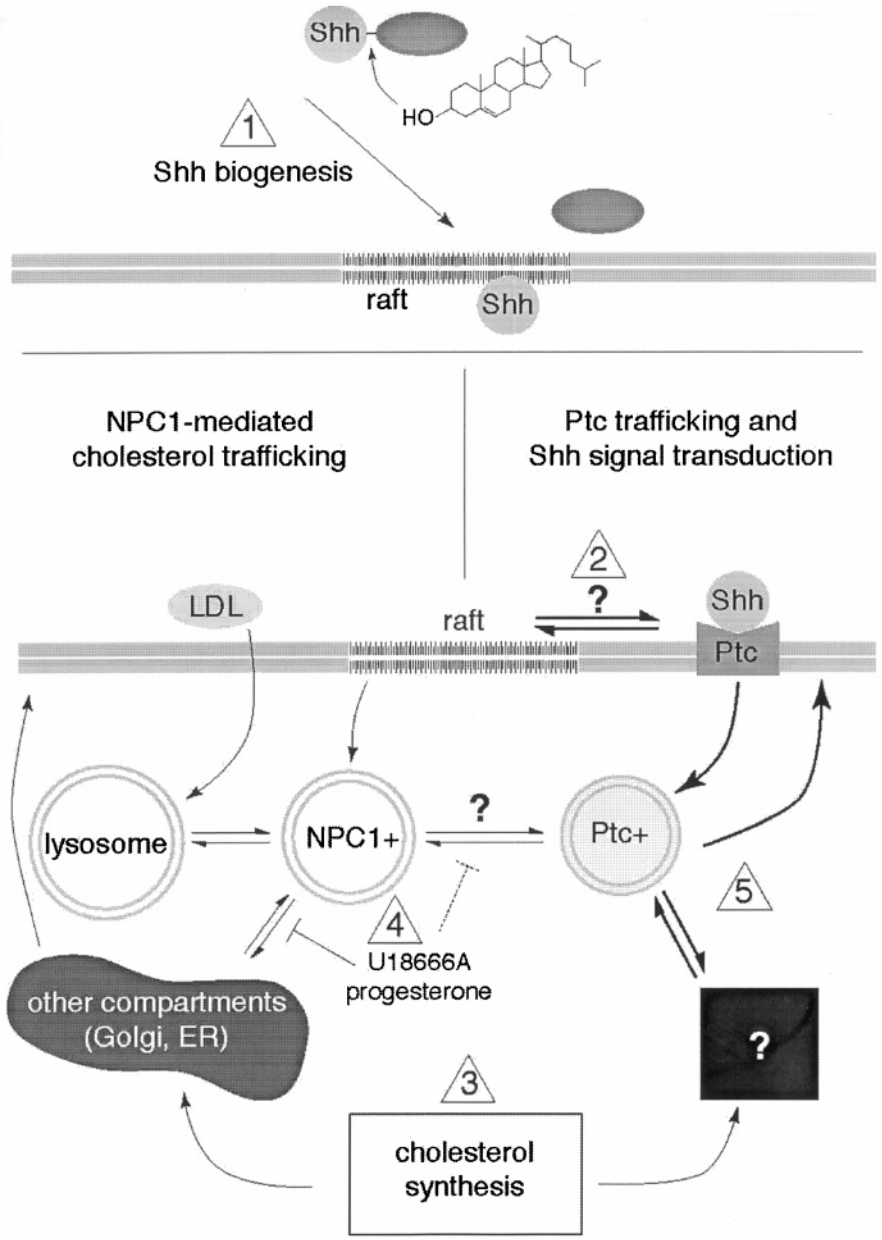

Figure 2. Model describing the potential sites for the involvement of cholesterol in Shh signaling, and potential targets (1–5) for teratogenic compounds affecting the pathway. (1) Cholesterol is required in cells that produce Shh because of its involvement in the autocatalytic processing of Shh. The cholesterol modified N-terminal fragment of Shh possesses all of the signaling activity, and associates with cholesterol-rich membrane rafts. However, Shh biogenesis is not the site of action for the agents discussed in the text. The role of cholesterol in Shh signal transduction within target cells appears to be novel and complicated, and four potential sites (2–5) are addressed. In the bottom portion of the figure, light arrows indicate intracellular movements of cholesterol, heavy arrows indicate known and hypothetical movements of Ptc. (2) Although rafts may be involved in proper sorting of Shh in source cells, a role for rafts in signal transduction in responsive cells has not been conclusively demonstrated. (3) The association of reduced 7DHCR activity with holoprosencephaly suggests that cyclopamine might also act through inhibition of late steps of cholesterol synthesis. Experiments described in the text suggest that this is not the case. The effect of reduced 7DHCR activity on Shh signal transduction may be mediated by a cholesterol precursor or metabolite thereof that has an activity similar to cyclopamine. (4) AY-9944 and cyclopamine were also proposed to secondarily inhibit Shh signal transduction by blocking intracellular cholesterol transport, which is known to be blocked by U18666A and progesterone. Cholesterol hypothetically could move via this pathway into a compartment (the Ptc + vesicle) where Shh signal transduction is regulated. (5) As suggested by its structural similarity to NPC1, Ptc may function in a vesicular trafficking pathway that is independently disrupted by cyclopamine, U18666A and progesterone. Ptc could potentially regulate Shh signal transduction by trafficking between the plasma membrane and a vesicular compartment (the Ptc + vesicle), or through an unidentified compartment (the black box).

which may be important for targeting Shh to its proper cellular destination. As described below, however, cyclopamine and the 7DHCR inhibitor AY-9944 have very clear effects on the Shh signaling pathway at the level of signal transduction in responsive cells.

Cholesterol-rich plasma membrane rafts are not required for Shh signal transduction

One hypothesis suggests that the action of cyclopamine might be related to a role in Shh signaling for rafts (fig. 2, point 2), the cholesterol-rich membrane microdomains implicated in many other signal transduction pathways (reviewed in [18, 21]). Rafts appear to be platforms for the spatial organization of receptors and second messenger effectors, many of which are post-translationally modified by lipids. Diverse signaling pathways appear to be mediated by raft-associated components, including signaling through many G-protein-coupled receptors.

Agents that bind cholesterol, such as the polyene antifungal filipin or modified cyclodextrins, have been shown to inhibit many raft-dependent processes in intact cells [22–25]. However, we found that these agents do not significantly affect Shh signal transduction in neural plate explants, despite inhibition of other raft-dependent processes [our unpublished observations]. Furthermore, cyclopamine had no effect on raft function in explants, indicating that it does not act as a general raft disrupter such as filipin. The results with cholesterol-binding agents indicate that plasma membrane rafts are neither the target of cyclopamine, nor required for Shh signal transduction, but do not preclude a role for an intracellular cholesterol-rich domain that is inaccessible to nonlytic concentrations of these agents.

As discussed below, other data suggest that an intracellular site may be involved in Shh signaling. However, we will first address the issue of whether cyclopamine and AY-9944 both affect Shh signaling secondary to inhibition of cholesterol synthesis.

The inhibition of Shh signaling by AY-9944 depends on tissue cholesterol source, but cyclopamine inhibition does not

An earlier study indicated that high concentrations of jervine (28 μM) partially inhibited a late step in cholesterol synthesis in COS-7 cells [26], resulting in an accumulation of a sterol intermediate (see fig. 1A). Although this suggests that jervine, cyclopamine and AY-9944 could block Shh signaling by the same mechanism (fig. 2, point 3), additional evidence does not support this interpretation. Most important, the effects of AY-9944 and cyclopamine or jervine in Shh signaling assays differ significantly with respect to the source of cholesterol. A fundamental feature of cholesterol homeostasis is that cells acquire cholesterol either by the uptake of cholesterol-rich lipoproteins or by de novo synthesis. In the presence of an adequate supply of exogenous cholesterol, de novo synthesis is suppressed, both at the level of the half-life of cholesterol biosynthetic enzymes, and transcription of genes encoding them [27, 28]. If the target of cyclopamine, like that of AY-9944, is in the cholesterol biosynthetic pathway, its presence should be dictated by the cholesterol supply. This premise is not supported by in vitro experiments.

Using a neural plate explant assay that bypasses effects on Shh biogenesis, cyclopamine, jervine and AY-9944 were found to inhibit the cellular response to Shh [19, 20]. However, AY-9944 failed to inhibit Shh signaling when exogenous cholesterol was added to the explant cultures either as lipoprotein or pure form dissolved in ethanol. Addition of cholesterol has no effect on the inhibition of Shh signaling by cyclopamine. Because the enzymes controlling cholesterol synthesis are downregulated by exogenous cholesterol, these findings strongly suggest that cyclopamine acts by a distinct mechanism, and not through effects on cholesterol synthesis. Like other key components of the cholesterol biosynthetic pathway, 7DHCR contains a 'sterol-sensing domain', and its levels are probably reduced in response to exogenous sterols [29]. Thus, AY-9944 has no effect in explants provided with cholesterol. We have extended these findings to a second in vitro Shh signaling assay, the induction of alkaline phosphatase in the mouse embryo-derived C3H10T1/2 cell line (fig. 3). As we observed for neural plate explants, AY-9944 significantly blocks Shh signaling in C3H10T1/2 cells only when they are cholesterol-starved, whereas cyclopamine blocks signaling in cells cultured with or without exogenous cholesterol.

These findings are entirely consistent with the in vivo teratogenicity of AY-9944 and cyclopamine. Cyclopamine causes holoprosencephaly both when administered to mammalian embryos by maternal ingestion and when applied directly to chick embryos in ovo. Chick embryos treated with cyclopamine also show defects in other Shh-dependent morphogenetic events, including patterning of the somites [20], determination of left-right asymmetry in the heart [J.P.I. and H.R., unpublished], and pancreas development [30]. In contrast, AY-9944 is known only to cause holoprosencephaly in mammalian embryos after maternal ingestion, and did not affect Shh-dependent patterning of the neural tube and somites when applied directly to chick embryos [20]. This is consistent with differences in cholesterol metabolism between early avian and mammalian embryos: chick embryos utilize cholesterol from preformed yolk particles, whereas mammalian embryos depend on

de novo synthesis within either embryonic or maternal tissue.

The effects of reduced 7DHCR activity on Shh signaling may result from the accumulation of a 'teratogenic' sterol precursor or metabolite, rather than simple reduction of cholesterol levels. Inhibitors of cholesterol synthesis, such as the statins (e.g. lovastatin), that block steps prior to formation of the first sterol intermediate (fig. 1A) are not associated with holoprosencephaly, and do not inhibit Shh signaling in explants [20]. The phenotypes associated with genetic defects in other late steps of cholesterol synthesis also support this interpretation. The *tattered* and *bare patches* mouse mutations were recently shown to affect loci encoding cholesterol biosynthetic enzymes [31, 32], and to produce an accumulation of different suites of sterol precursors. The *tattered* mouse corresponds to Conradi-Hunermann-Happle syndrome, or X-linked chondrodysplasia punctata [33], a form of dwarfism in humans. The features of this disorder have only minimal overlap with those of Smith-Lemli-Opitz syndrome, and are not obviously associated with failure of Shh-dependent patterning [31–34]. Therefore, a metabolite unique to loss of 7DHCR activity may be responsible for holoprosencephaly in Smith-Lemli-Opitz syndrome.

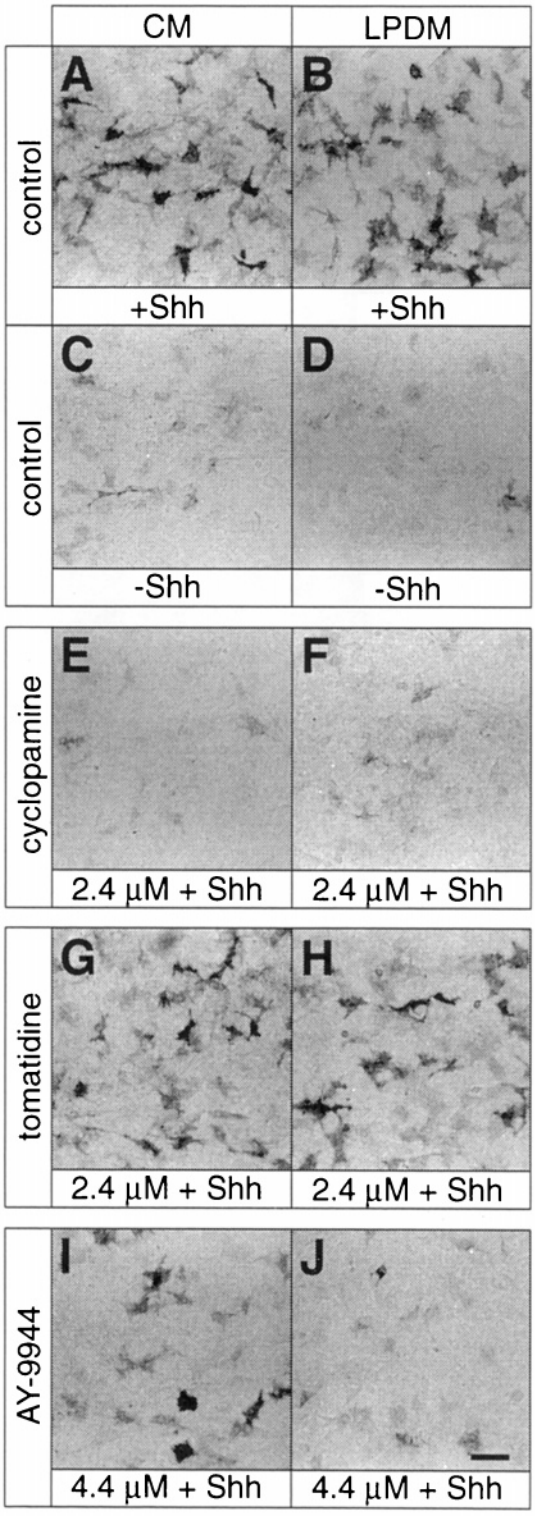

Figure 3. Cholesterol-independent inhibition of Shh signaling in C3H10T1/2 cells by cyclopamine. C3H10T1/2 cells were passaged either in complete medium containing 10% serum (CM) or in medium containing 10% lipoprotein-deficient serum (LPDM) to deplete cholesterol stores and activate de novo cholesterol synthesis. Cells were then cultured for 3 days in the presence of recombinant Shh-N in either CM or LPDM, then fixed and processed for alkaline phosphatase histochemistry. The images show randomly selected fields of confluent cells, with alkaline phosphatase-positive (black precipitate) and -negative cells intermixed. Only a percentage of cells typically demonstrate this response to Shh. Note that the response to Shh-N is essentially identical in CM (*A*, *C*) or LPDM (*B*, *D*). Cyclopamine at 2.4 μM completely blocks the response to Shh-N in either CM (*E*) or LPDM (*F*). Cyclopamine inhibition of the C3H10T1/2 response begins at 0.24 μM and is maximal at 0.8 μM (not shown). The nonteratogenic but structurally similar steroidal alkaloid tomatidine has no effect on the C3H10T1/2 response at 2.4 μM in either medium (*G*, *H*). At 4.4 μM AY-9944 has minimal inhibitory effect on the Shh-N response in cells cultured in CM (*I*), but produces a nearly complete inhibition of Shh signaling in cells cultured in LPDM (*J*). Scale bar is 50 μM.

Is there a link between Shh signaling and cholesterol transport?

Inhibition of cholesterol transport (fig. 2, point 4) was suggested as a common basis for the effects of both cyclopamine and AY-9944 on Shh signaling [19]. High concentrations of AY-9944 were found to inhibit the intracellular movements of cholesterol [35], an activity unrelated to the inhibition of 7DHCR. This effect is

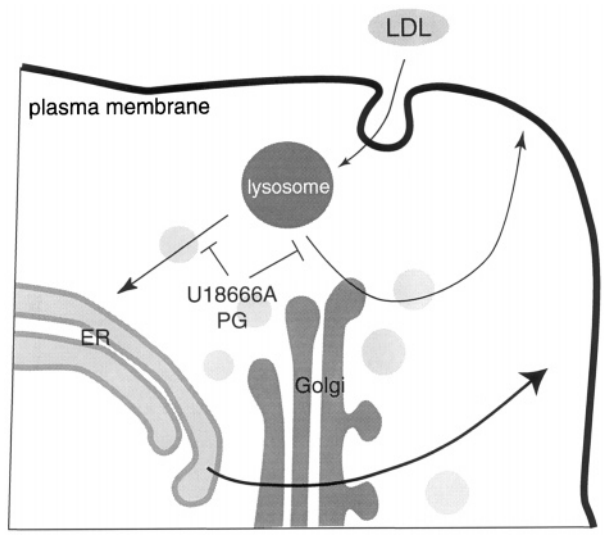

Figure 4. Some important pathways for the intracellular transport of cholesterol. LDL particles internalized by receptor-mediated endocytosis are processed in late endosomes/lysosomes. Free cholesterol liberated from LDL is then transported from lysosomes to other cellular sites. Major pathways that involve vesicular transport (light arrows) are to the plasma membrane via the Golgi, and a putative direct route from lysosomes to the ER. Both of these pathways are blocked by U18666A and progesterone (PG), and are defective in cells carrying NPC1 mutations. Cholesterol synthesized in the ER is transported to the plasma membrane by a different pathway (heavy arrow) not affected by these compounds or NPC1 mutations.

characteristic of a large group of mostly unrelated compounds, which includes several hydrophobic amines (e.g. imipramine, chloroquine, AY-9944), progesterone and the steroidal amine U18666A [36–41]. To illustrate this activity, some major aspects of the intracellular trafficking of cholesterol are modeled in figure 4 (see [42] for review).

LDL-cholesterol obtained by endocytosis is hydrolyzed in late endosomes or lysosomes, and free cholesterol transported to intracellular sites via several incompletely characterized pathways. Several lines of evidence suggest that a major pathway is mediated by a vesicular process and involves the Golgi apparatus. Any excess cholesterol is delivered from lysosomes or the plasma membrane to the endoplasmic reticulum (ER), where it is esterified for storage. The ER contains a sensor mechanism which 'measures' the amount of cholesterol, such that de novo cholesterol synthesis is activated if delivery of sterols is curtailed. Cholesterol synthesized in the ER moves to the plasma membrane by a pathway different from the influx of cholesterol. Hydrophobic amines and steroids block the inward flux of cholesterol from lysosomes and/or the plasma membrane. Treatment of cells with these agents then results in the accumulation of cholesterol in lysosomes and modulation of enzymes involved in cholesterol synthesis and esterification [35–39, 41]. Importantly, these effects are indistinguishable from the cellular phenotype associated with the Niemann-Pick type C (NP-C) lysosomal storage disorder, which results from mutations in NPC1. Furthermore, as discussed below, recent studies indicate that the NPC1 protein plays a central role in the intracellular trafficking of cholesterol, and may be a target of hydrophobic amines and steroids.

Two findings led to the proposal that the inhibition of Shh signaling by both AY-9944 and cyclopamine was secondary to blockade of the cholesterol transport pathway(s) described above [19]. First, U18666A, progesterone and imipramine (all drugs that block cholesterol transport) were reported to block Shh signaling in the explant assay. Second, cyclopamine and jervine (both hydrophobic amines) were reported to block cholesterol transport as measured by effects on esterification. However, a more detailed examination of these effects do not support this hypothesis [43]. We found no correlation between a compound's potency in blocking Shh signaling and its potency in blocking cholesterol transport.

In our hands, U18666A and progesterone proved to be weak inhibitors of Shh signaling in neural plate explants, and do not block all responses to Shh at subtoxic concentrations (2.4 and 30 μM, respectively). In contrast, cyclopamine completely inhibits all measurable responses to Shh at very low concentrations (0.1 μM) that have no toxic effect on neural plate cells. Furthermore, concentrations of cyclopamine that completely block Shh signaling did not affect cholesterol transport in explants, whereas concentrations of U18666A and progesterone that blocked explant cholesterol transport only partially inhibited Shh signaling. Finally, we found that subtle changes in steroidal alkaloid structure dramatically affect the ability to inhibit Shh signaling in explants, corresponding with the well-known structure/activity relationships for the induction of holoprosencephaly by steroidal alkaloids in vivo [10, 11]. In contrast, changes in steroidal alkaloid structure were not associated with changes in potency for the inhibition of cholesterol transport: all steroidal alkaloids had similar effects on cholesterol transport, with maximal inhibition occurring at concentrations above 10 μM. We therefore believe that the inhibition of cholesterol transport by high concentrations of cyclopamine and related steroidal alkaloids are side effects unrelated to the inhibition of Shh signal transduction.

A more parsimonious explanation of the effects of U18666A and progesterone on Shh signaling is achieved by a closer examination of the relationship of Ptc to NPC1, provided below. However, we believe that AY-9944, cyclopamine, U18666A and progesterone still

cannot be grouped as acting through an identical mechanism. Cyclopamine appears to be a uniquely potent Shh antagonist.

Sequence homology and similar pharmacology suggest a functional relationship between Ptc and NPC1

A mechanistic alternative for the action of cyclopamine is suggested by revisiting cholesterol transport, NP-C disease and NPC1. The members of the Ptc family, Ptc-1 and Ptc-2, are structurally similar to NPC1 [44, 45]. Ptc and NPC1 both are large polytopic membrane proteins with 12 and 13 putative transmembrane segments, respectively. All of the transmembrane segments of Ptc have a homologous segment in NPC1, including five segments (II–VI) that constitute a 'sterol-sensing domain' that is also present in three additional proteins involved in cholesterol metabolism (including 7DHCR) [29, 46, 47]. The sequence identity between Ptc and NPC1 is even greater for putative transmembrane segments VIII–XII of Ptc, and includes a short extramembrane section near the end of the second extracellular loop of Ptc, just proximal to segment VIII.

As described above, mutations in NPC1 result in cellular defects in cholesterol transport that are closely mimicked by treating cells with drugs such as U18666A, progesterone and high concentrations of cyclopamine. However, Shh signaling must be intact in cells lacking functional NPC1, because there are no morphologic effects of NPC1 mutations in both humans and the mouse, although defects in cholesterol transport can be detected at fetal stages [48–51]. Why would Shh signaling be affected by pharmacologic disruption of NPC1-mediated cholesterol transport, but not by genetic disruption of NPC1? A simpler explanation of the data is that Ptc and NPC1 are structurally similar, and therefore have similar pharmacologic sensitivities. Although Ptc could 'sense' the flow of cholesterol into a compartment where it functions, or interact directly with NPC1, and thereby is affected by drugs like U18666A, neither of these explanations seem as satisfactory. Moreover, we have also tested other agents that are standard tools for the manipulation of cellular cholesterol pools, and found that the only compounds that significantly affect Shh signaling are those that also induce the NP-C phenocopy.

The specific effects of U18666A and progesterone on NPC1 suggest how these drugs and cyclopamine could affect Ptc function and Shh signaling. In normal cells, NPC1 is found on a population of cytoplasmic vesicles with characteristics of late endosomes [52, 53]. In cells treated with drugs such as U18666A and progesterone, cholesterol accumulates in vesicles with lysosomal characteristics. Under these conditions, NPC1 is found to colocalize with the accumulated cholesterol, indicating that it has become entrapped within lysosomes with its cargo [52]. Therefore, it is possible that the normal NPC1 + vesicle regulates cholesterol transport through a 'kiss-and-run' cycle with lysosomes, and that U18666A and progesterone disrupt the fission of NPC1 + vesicles from the lysosome. Furthermore, the compartment in which cholesterol accumulates in both U18666A-treated and NP-C cells is enriched with the unique lipid, lysobisphosphatidic acid (LBPA) [54]. LBPA is found only in the core of these vesicles, and treatment with U18666A alters the ultrastructural appearance of this domain. Additionally, internalization of an anti-LBPA monoclonal antibody by live cells resulted in the same changes induced by U18666A, including the NP-C phenocopy. Analysis of NPC1 mutant proteins suggests that NPC1 function may involve an interaction with the LBPA-enriched vesicle core. Several mutations, including those in the sterol-sensing domain, result in a protein that localizes at the limiting membrane of the cholesterol-accumulating compartment but fails to extract cholesterol from the core [55, 56]. NPC1 could conceivably undergo a U18666A-sensitive interaction with LBPA itself (as perhaps suggested by the effects of anti-LBPA antibodies), or with another protein that resides in that compartment.

In this light the extensive homology between Ptc and NPC1 is even more intriguing. Ptc might function in a vesicular trafficking process involving similar compartments that is disrupted by drug treatment independently of, or in parallel with, NPC1 trafficking (fig. 2, point 5). In the *Drosophila* embryo, a large fraction of Ptc is found on cytoplasmic vesicles that are derived by dynamin-mediated endocytosis [57]. Some of these vesicles have the characteristics of late endosomes, with Ptc immunoreactivity associated with electron-dense multivesicular structures. Because Hh colocalizes with Ptc in vesicular structures at the light microscopic level [58], it is likely that Ptc mediates the endocytosis of Hh. However, it is unknown whether Hh signaling occurs at the plasma membrane, or whether these vesicular structures are involved in signaling. Nevertheless, the distribution of Ptc indicates that its trafficking may intersect with that of NPC1. Because each of the 12 transmembrane segments of Ptc has a homologous segment in NPC1 [44, 45], it is possible that both proteins reside in similar membrane environments, and that both proteins are affected by similarly lipophilic compounds. NPC1 and Ptc both might undergo similar protein-protein interactions with other partners along the pathway.

We hypothesize that a Ptc-mediated vesicular trafficking step is crucial for Shh signal transduction, as described in further detail below. This process may be affected by U18666A and progesterone, but exquisitely

sensitive to cyclopamine. It is possible that Ptc regulates Shh signal transduction via relatively cholesterol-rich vesicles (rather than a plasma membrane raft), and the drugs disrupt the interaction of Ptc with this compartment or a component within. Could the LBPA-enriched compartment be involved in both Shh signaling and cholesterol transport? The assessment of the effects of anti-LBPA antibodies on Shh signaling may shed further light on the Ptc/NPC1 relationship.

Reductions in 7DHCR activity may produce a sterol precursor/metabolite with an activity similar to cyclopamine, but there is no direct evidence for this yet. Although high concentrations of AY-9944 could have the same affect on Ptc as U18666A, progesterone, and cyclopamine, this activity is irrelevant for holoprosencephaly resulting from 7DHCR mutations. There is no known cholesterol trafficking defect associated with Smith-Lemli-Opitz syndrome and 7DHCR mutations, although we would predict that high levels of 7-dehydrocholesterol or other precursors might also affect NPC1 function. Effects of the *megalin* mutation on cholesterol transport also have not been demonstrated, and it is still unclear how megalin fits into this picture. Megalin has been shown to be a receptor for a variety of ligands other than lipoproteins [59–63]. The recent finding of cholesterol-independent functions for LDL-receptor family members in reelin/disabled signaling [64] indicates that megalin could potentially play a role in Shh signaling entirely unrelated to cholesterol. Megalin is the only member of the LDL-receptor family that is found in late endosomal compartments [65], suggesting that its trafficking could overlap that of Ptc.

A revised model for Shh signal transduction

The pharmacology of Shh signaling antagonists and the Ptc/NPC1 relationship suggest a revised model for early steps in the pathway that incorporates Ptc-mediated vesicular trafficking (fig. 5). Ptc is an unusual receptor: it has no inherent signaling activity, is related to a protein involved in vesicular trafficking and also apparently functions as an inducible negative regulator of Hh signaling. In the current model, Ptc acts as the ligand-binding subunit of the Shh receptor [66, 67] and regulates the activity of Smoothened (Smo), which functions as the signaling subunit and is related to G-protein-coupled receptors [68, 69]. In the absence of ligand, Ptc inhibits the constitutive signaling activity of Smo; the inhibition is released upon ligand binding [70, 71]. Although Ptc and Smo can be coimmunoprecipitated from cotransfected cells [66, 71], it is not known whether the release of Smo inhibition by Ptc involves a physical uncoupling, or by permitting the access of Smo to another activating factor. If cyclopamine acts at this level, it would have to freeze or stabilize the inhibitory activity of Ptc. Could this activity involve vesicular trafficking?

By incorporating a Ptc-mediated trafficking process, some of the inconsistencies in the current model are more easily explained, such as the dual roles of Ptc as a receptor subunit and inducible negative regulator of Hh signaling. One interpretation of the genetic data is that Ptc and Smo compete for Hh binding; however, the failure to detect a biochemical interaction between Smo and Hh does not support this. Another possibility is

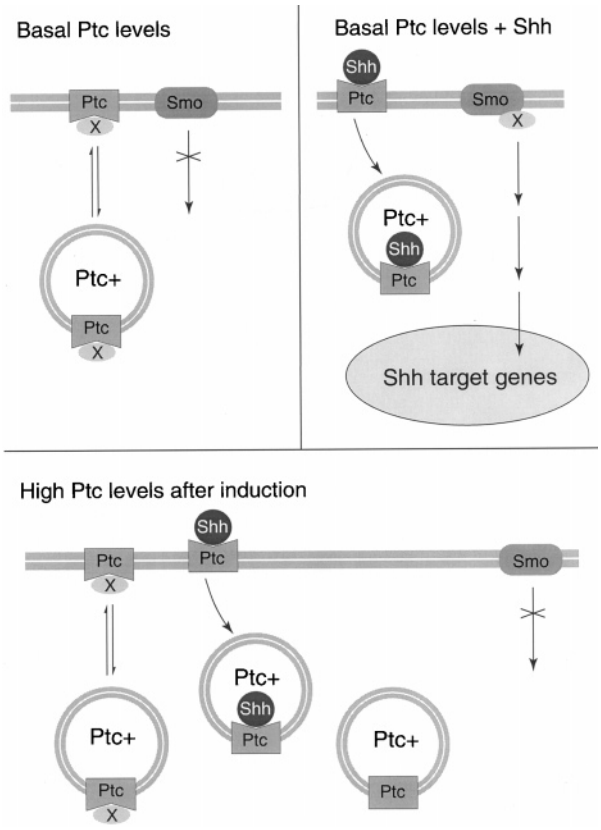

Figure 5. A model for Shh signal transduction incorporating a vesicular trafficking process mediated by Ptc. Ptc and Smo could compete for an intracellular second messenger effector (*X*), which might bind Ptc at the plasma membrane or on a cytoplasmic vesicle. The model is based on the assumption that Shh can bind to Ptc with or without factor X present. Regulation of signal transduction could be achieved simply by varying the ratio of Ptc to factor X. Binding of Shh to Ptc at the plasma membrane could alter the affinity of Ptc for factor X, such that Smo competes more effectively for binding. Factor X bound to Smo activates intracellular signaling. At the same time, Ptc mediates the internalization of Shh to initiate downregulation of signaling. After induction of high levels of Ptc by Shh Ptc could be present in three states: bound to factor X, bound to Shh or free. Signaling would thus be further downregulated due to both higher competition of Ptc for factor X and increased removal of Shh from the plasma membrane by Ptc that is not associated with factor X.

that Ptc and Smo compete for binding to a second-messenger effector (none of which has been identified). That effector could be included as 'cargo' on a Ptc + vesicle that cycles between the plasma membrane and cytoplasm; binding of Hh could induce the release of that cargo, allowing the effector to interact with Smo. In the absence of Ptc, Smo would have no competition for the effector and signal constitutively, whereas signaling would not occur after the induction of high levels of Ptc by Hh. In analogy with NPC1, cyclopamine could induce the entrapment of Ptc with the effector, thereby indirectly blocking signal transduction through Smo. A *Drosophila* Ptc mutant that fails to inhibit Smo, but still mediates the internalization of Hh [72], is consistent with this model. Proline-rich regions in the C-termini of both Ptc and Smo could potentially mediate protein-protein interactions with the putative effector. This model can be tested by characterizing the cellular distributions of Ptc and Smo, how they may be affected by cyclopamine, and by determining whether cyclopamine can inhibit the constitutive signaling of Smo.

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