letter

A defective response to Hedgehog signaling in disorders of cholesterol biosynthesis

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Smith–Lemli–Opitz syndrome (SLOS), desmosterolosis and lathosterolosis are human syndromes caused by defects in the final stages of cholesterol biosynthesis. Many of the developmental malformations in these syndromes occur in tissues and structures whose embryonic patterning depends on signaling by the Hedgehog (Hh) family of secreted proteins. Here we report that response to the Hh signal is compromised in mutant cells from mouse models of SLOS and lathosterolosis and in normal cells pharmacologically depleted of sterols. We show that decreasing levels of cellular sterols correlate with diminishing responsiveness to the Hh signal. This diminished response occurs at sterol levels sufficient for normal autoprocessing of Hh protein, which requires cholesterol as cofactor and covalent adduct. We further find that sterol depletion affects the activity of Smoothened (Smo), an essential component of the Hh signal transduction apparatus.

The role of cholesterol in Hh protein biogenesis suggested that impaired Hh autoprocessing might underlie some of the developmental abnormalities in SLOS (Fig. 1 and Table 1; ref. 1). An additional role for cholesterol in Hh signal response was suggested by the observation that cyclopamine and jervine, teratogenic plant alkaloids that block Hh signaling, also inhibit cholesterol transport and synthesis[2,3]. But cyclopamine has since been shown to specifically inhibit Hh signaling by binding to a pathway component[4], and the doses of these alkaloids required to inhibit Hh signaling are lower than those required to block cholesterol transport (ref. 5 and M.K.C., unpublished data).

To determine how cholesterol may affect Hh signaling in embryonic development, we exposed chick embryos to cyclodextrin, a cyclic oligosaccharide that forms non-covalent complexes with sterols[6] and can be used to extract and deplete cholesterol from living cells[7]. Cyclodextrin treatment caused variable loss of the frontonasal process and other midline structures (Fig. 2a), and the spectrum of facial defects was similar to that resulting from exposure to the Hh-pathway antagonist jervine[3]. The most severely affected embryos developed a proboscis-like structure that phenocopies the nasal rudiments of mouse embryos that are homozygous with respect to mutations in the gene Sonic hedgehog (*Shh*; ref. 8).

Fig. 1 Cholesterol biosynthesis and Hh pathway. *a*, Autosomal recessive disorders with multiple developmental anomalies are associated with three different enzymatic defects (represented by solid colored lines) in the final steps of the cholesterol biosynthesis pathway. SLOS results from defects in DHCR7 (red line), lathosterolosis from defects in SC5D (green line) and desmosterolosis from defects in 3-β-hydroxysterol-Δ^{24}-reductase (yellow line). Steps in sterol synthesis not shown are indicated by multiple arrows. *b*, After cleavage of the signal sequence (black box), the Hh precursor undergoes an internal cleavage reaction mediated by sequences in the C-terminal autoprocessing domain (white box). Cholesterol participates in the reaction and remains esterified to the newly formed C terminus of the signaling domain (shaded box). Fully processed, secreted Hh proteins (designated HhNp, p for processed) also receive an N-terminal palmitoyl group in a reaction requiring the acyltransferase Skinny hedgehog[30]. The response to Hh is regulated by two transmembrane proteins, Ptch (in red) and Smo (in green). Ptch suppresses the activity of Smo, and Hh binding to Ptch inhibits this function (–), leading to Smo activation of a transcriptional response through the Gli family of transcription factors. *Ptch* is a transcriptional target of Hh signaling and thus forms a negative feedback loop that ensures adequate regulation of Smo in the absence of Hh. Multiple arrows indicate the participation of a complex of Hh signaling components not shown.
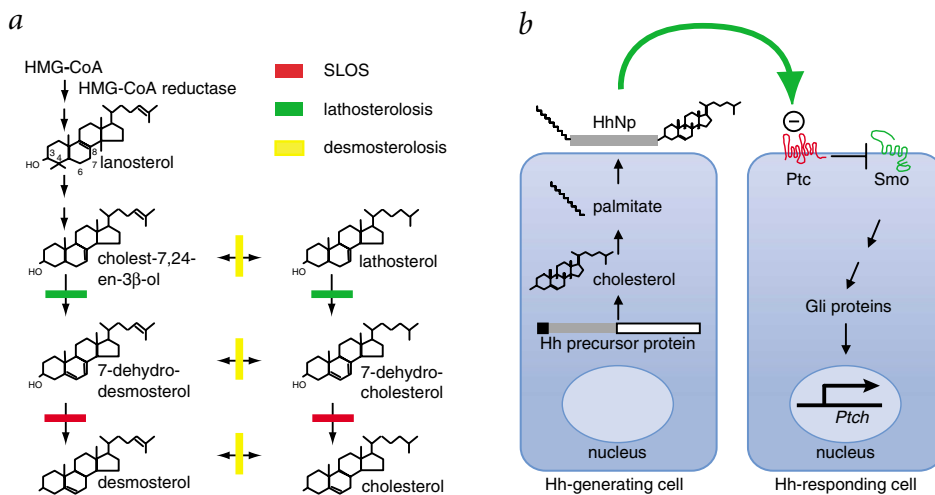
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**Table 1 • Developmental malformations in tissues patterned by Hh signaling**

Hh family member	Source of Hh signal	Target tissue	Normal role of Hh signaling	Consequence of disrupted Hh signaling	PHS[25]	SLOS[25]	Lath[26]	Des[27]
	Prechordal plate mesendoderm and ectoderm of facial processes	Ventral neuroectoderm and mesenchyme of facial processes	Formation of midline forebrain and facial structures	Holoprosencephaly	+	+	−	+
Sonic hedgehog	Posterior limb bud mesenchyme	Limb bud mesenchyme and ectoderm	Patterning of the autopod and zeugopod	Post-axial polydactyly and syndactyly	+ +	+ +	+ +	
	Lung bud epithelium	Lung bud mesoderm	Branching morphogenesis of lung	Unilobular lungs	+	+		
Indian hedgehog	Prehypertrophic chondrocytes	Chondrocytes and osteoblasts	Regulation of endochondral bone growth	Rhizomelia	+	+		
	Colonic epithelium	Neural crest cells (?)	Development of the enteric nervous system	Aganglionic megacolon (Hirschsprung disease)	+	+		
Desert hedgehog	Sertoli cells	Leydig cells	Development of the male gonad	Cryptorchidism/ ambiguous genitalia or hypospadias	+/+	+/+	+/+	+/−

Overlap of developmental anomalies in tissues patterned by members of the mammalian family of Hh signaling proteins[28] among individuals with Pallister–Hall syndrome (PHS), SLOS, lathosterolosis (Lath) and desmosterolosis (Des). PHS is an autosomal dominant disorder associated with mutations in *GLI3* that reduce transcriptional activation of Hh pathway targets[29].

We further investigated Shh signaling in embryonic tissues by exposing chick neural-plate explants to recombinant Shh protein (ShhN, 30 nM) in the presence of cyclodextrin (Fig. 2*b*). The response to this level of ShhN protein was uniform production of HNF3β (hepatocyte nuclear factor 3β or forkhead box A2; ref. 3), an indicator of floor plate fate. This high-threshold response was blocked in the presence of 400 μM cyclodextrin and replaced by nearly uniform expression of ISL1, an intermediate-threshold

Fig. 2 Cyclodextrin treatment inhibited Shh signaling. *a*, Cyclodextrin treatment of chick embryos *in ovo* caused holoprosencephaly. Scanning electron micrographs of facial features at embryonic day 5 of a control (control) chick embryo and of embryos exposed to 375 μM cyclodextrin (CD). Cyclodextrin treatment at the early-primitive-streak stage led to variable loss of midline tissues, primarily the frontonasal process (fnp), and approximation or fusion of paired lateral structures, such as the lateral nasal processes (lnp), optic vesicles (opt) and the maxillary (mx) and mandibular (mn) processes. The slanting nostrils of less severely affected chick embryos treated with cyclodextrin resembled the anteverted nares characteristic of SLOS. Complete loss of the frontonasal process and subsequent fusion of the lateral nasal processes (marked by the asterisk at the top border of the lateral nasal process) led to the formation of a proboscis with a single nasal pit, positioned above fused optic vesicles. *b*, Cyclodextrin treatment inhibited the cellular response to Shh protein. Confocal microscope images of stage 9–10 chick embryo ectoderm dissected from a region of the neural plate intermediate to the notochord and roof plate and just rostral to Hensen's node. Explants were cultured for 48 h in collagen and stained for HNF3β (red) or ISL1 (green). Neural progenitor cells explanted from this position and at this developmental stage did not express markers of either floor-plate (HNF3β) or motor-neuron (ISL1) cell fates. Addition of 30 nM ShhN at the onset of culture induced a high-threshold response indicated by the expression of HNF3β. In the presence of 400 μM cyclodextrin, however, HNF3β induction was completely blocked. ISL1 expression is indicative of an intermediate-threshold response and was not present with 30 nM ShhN. But, in the presence of 200 and 400 μM cyclodextrin, partial inhibition of the high-threshold signaling dose of 30 nM ShhN produced the intermediate-threshold response of ISL1 expression. *c*, Cyclodextrin treatment did not inhibit a BMP-mediated signaling event. Dorsal neural plate progenitor cells and an endogenous source of BMP, the adjacent epidermal ectoderm, were dissected from a region just rostral to Hensen's node in stage 9–10 chick embryos. After 48 h of culture in the presence of 400μM cyclodextrin, phase contrast and confocal microscope images show that neural crest–like, HNK1-positive cells migrated from the explant into the surrounding collagen.
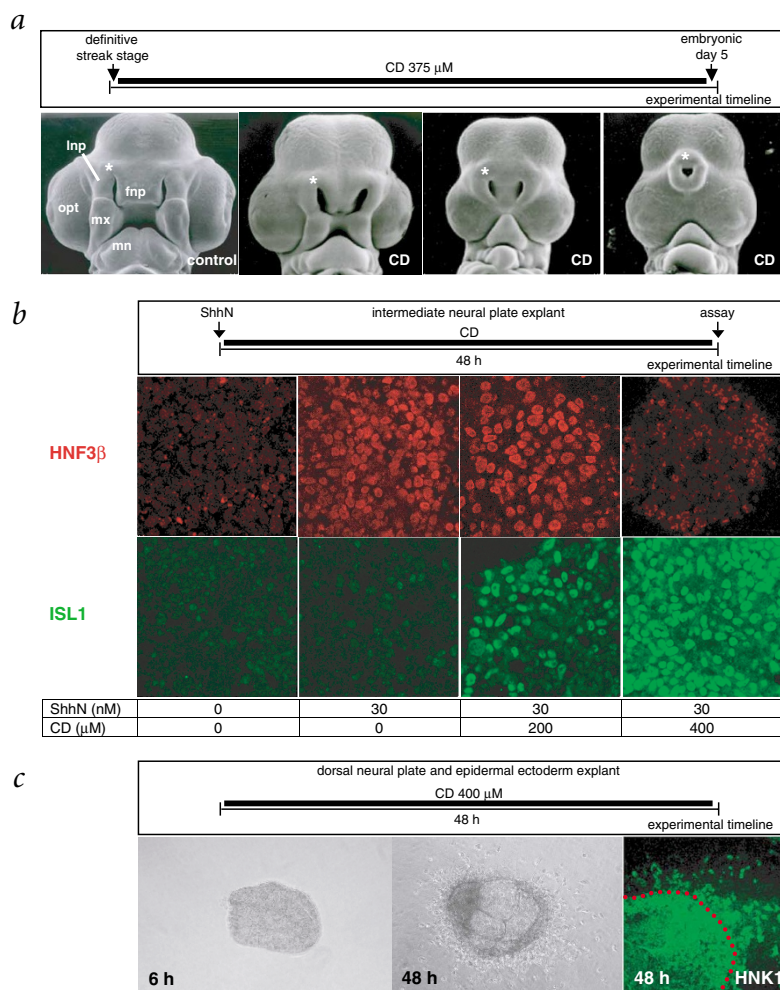
Fig. 3 Cells defective in cholesterol biosynthesis did not respond to Shh. *a*, Shh autoprocessing proceeded to completion in *Dhcr7*^{−/−} and *Sc5d*^{−/−} MEFs at low cholesterol levels. Embryonic fibroblasts generated from *Dhcr7*^{+/−} (lanes 4–6), *Dhcr7*^{−/−} (lanes 7–9), *Sc5d*^{+/−} (lanes 13–15) and *Sc5d*^{−/−} (lanes 16–18) mice were transiently transfected with a full-length Shh expression construct and cultured in full serum (fetal bovine serum, FBS; lanes 4, 7, 13 and 16), lipid-depleted serum (LDS; lanes 5, 8, 14 and 17) or lipid-depleted serum after 30 min of treatment with cyclodextrin (CD/LDS; lanes 6, 9, 15 and 18). Shh was efficiently processed under all culture conditions as there was no detectable accumulation of precursor protein (M_r = 45 kDa). Purified ShhNp (lanes 2 and 11; M_r = 19.5 kDa) was cell-associated and migrated faster than unprocessed ShhN protein (lanes 1 and 10) collected from the medium of cultured cells transfected with a construct carrying an open reading frame truncated after Gly198 (both ShhNp and ShhN are loaded in lanes 3 and 12), indicating that ShhNp from the treated MEFs (lanes 4–9) probably carried a sterol adduct. *b*, Response of *Dhcr7*^{−/−} and *Sc5d*^{−/−} MEFs to Shh protein was inhibited at low cholesterol levels. The effect of cholesterol depletion on responsiveness of *Dhcr7*^{+/−} (gray bars), *Dhcr7*^{−/−} (maroon bars), *Sc5d*^{+/−} (gray bars) and *Sc5d*^{−/−} (green bars) MEFs to Shh signaling was assayed by transfection with a Hh-responsive firefly luciferase reporter and treatment with purified ShhNp

(10 nM). MEFs were transfected at sub-confluent densities, cultured to maximum cell density and then induced with ShhNp either in culture medium containing fetal bovine serum (full) or lipid-depleted serum. Some of the cells cultured in lipid-depleted serum were stripped of surface cholesterol with a 30-min exposure to cyclodextrin just before ShhNp induction. For each culture condition, normalized firefly luciferase activity from control and ShhNp treated cells was used to calculate the relative induction, expressed as a percentage of the Shh induction achieved in MEFs cultured in full serum. Bars represent the standard error from quadruplicate experiments.
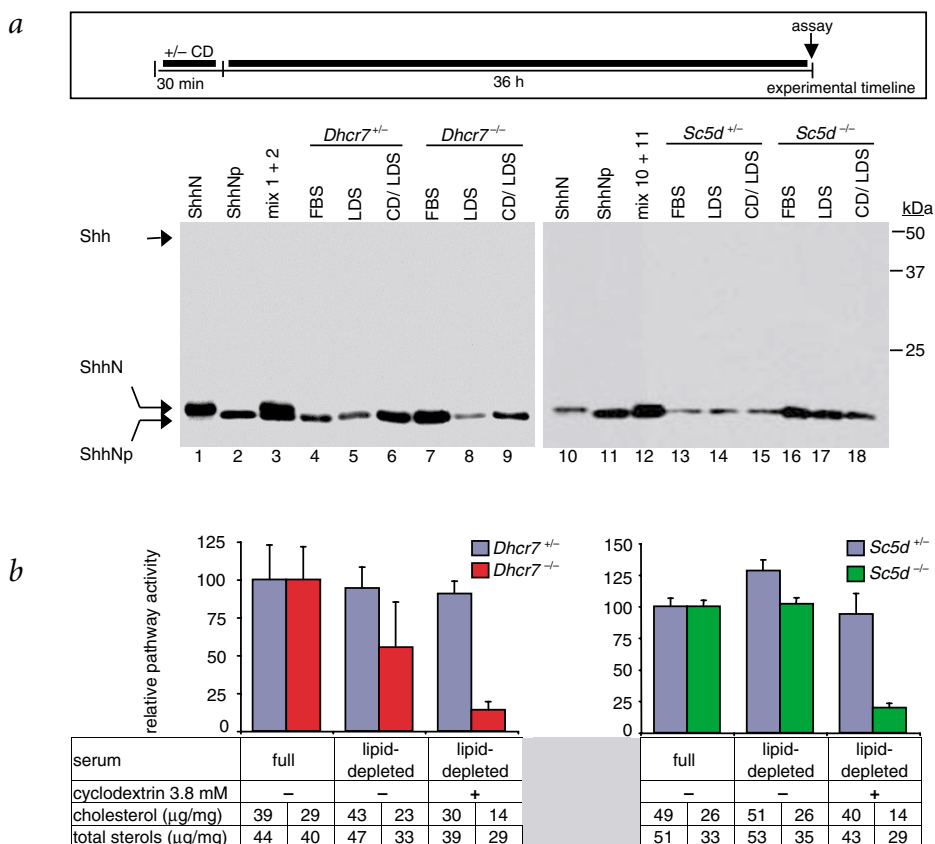
response indicative of motor-neuron fate. At 200 µM cyclodextrin, both HNF3β and ISL1 were expressed, suggesting that lower levels of cyclodextrin were less inhibitory. As active ShhN protein was exogenously supplied, this dose-dependent inhibition suggests that sterol deficits affect response to the Hh signal. Furthermore, this effect seemed to be specific, as treatment with 400 µM cyclodextrin alone did not inhibit BMP-induced migration of neural-crest cells[9] (Fig. 2*c*).

Hh protein biogenesis involves internal cleavage and covalent addition of cholesterol through an autoprocessing reaction[1]. Cyclodextrin treatment of cultured cells has previously been reported to interfere with Shh autoprocessing[10], an effect distinct from inhibition of response that we observed. To further investigate whether signal production or signal response is the prevailing inhibitory mechanism in cholesterol synthesis disorders, we established embryonic fibroblast cell lines from mouse models of SLOS[11] and lathosterolosis and examined Shh signal biogenesis and response in parallel under identical culture conditions.

Shh protein was efficiently processed in mouse embryonic fibroblasts (MEFs) lacking 7-dehydrocholesterol reductase (Dhcr7) or lathosterol 5-desaturase (Sc5d) enzymes (models of SLOS and lathosterolosis, respectively; see Fig. 1*b*), with no observable effect of transient cyclodextrin treatment or growth in lipid-depleted culture medium (Fig. 3*a*). All of the processed N-terminal product (ShhNp) from mutant cultures was cell-associated and had an electrophoretic mobility suggestive of sterol modification (Fig. 3*a*). The autoprocessing reaction probably

proceeds to completion because cholesterol levels are only reduced by roughly 50% under the conditions used for depletion (Fig. 3*b*) and because 7-dehydrocholesterol and lathosterol both participate efficiently as sterol adducts in the Shh processing reaction[3]. Likewise, cholesterol levels are reduced but never absent in the serum of individuals with SLOS[12].

In contrast with their normal Shh autoprocessing, MEFs with mutations in *Dhcr7* and *Sc5d* had clear deficiencies in their ability to respond to Shh signal when transiently treated with cyclodextrin and grown in lipid-depleted culture medium (Fig. 3*b*). These results indicate that signal response is more sensitive than is signal biogenesis to perturbations of cholesterol homeostasis. Cells heterozygous with respect to the mutations in *Dhcr7* and *Sc5d* maintained a normal response to ShhNp stimulation under all growth conditions (Fig. 3*b*), presumably because there was sufficient synthetic activity from the functional allele. In these experiments, the initial transient cyclodextrin treatment is needed to reduce sterol levels to below 40 µg mg^{−1} protein to affect pathway response (Fig. 3*b*).

To further investigate Hh signal response in other cultured cells, we tested the ability of pharmacological interventions to mimic the effects of genetic defects in sterol biosynthesis. Continuous treatment with 500 µM cyclodextrin produced about 50% inhibition of Shh signal response in NIH3T3 cells, and 2 mM cyclodextrin produced nearly complete inhibition (Fig. 4*a*). Transient cyclodextrin treatment for 30 minutes before exposure to ShhNp, however, did not affect response, even at
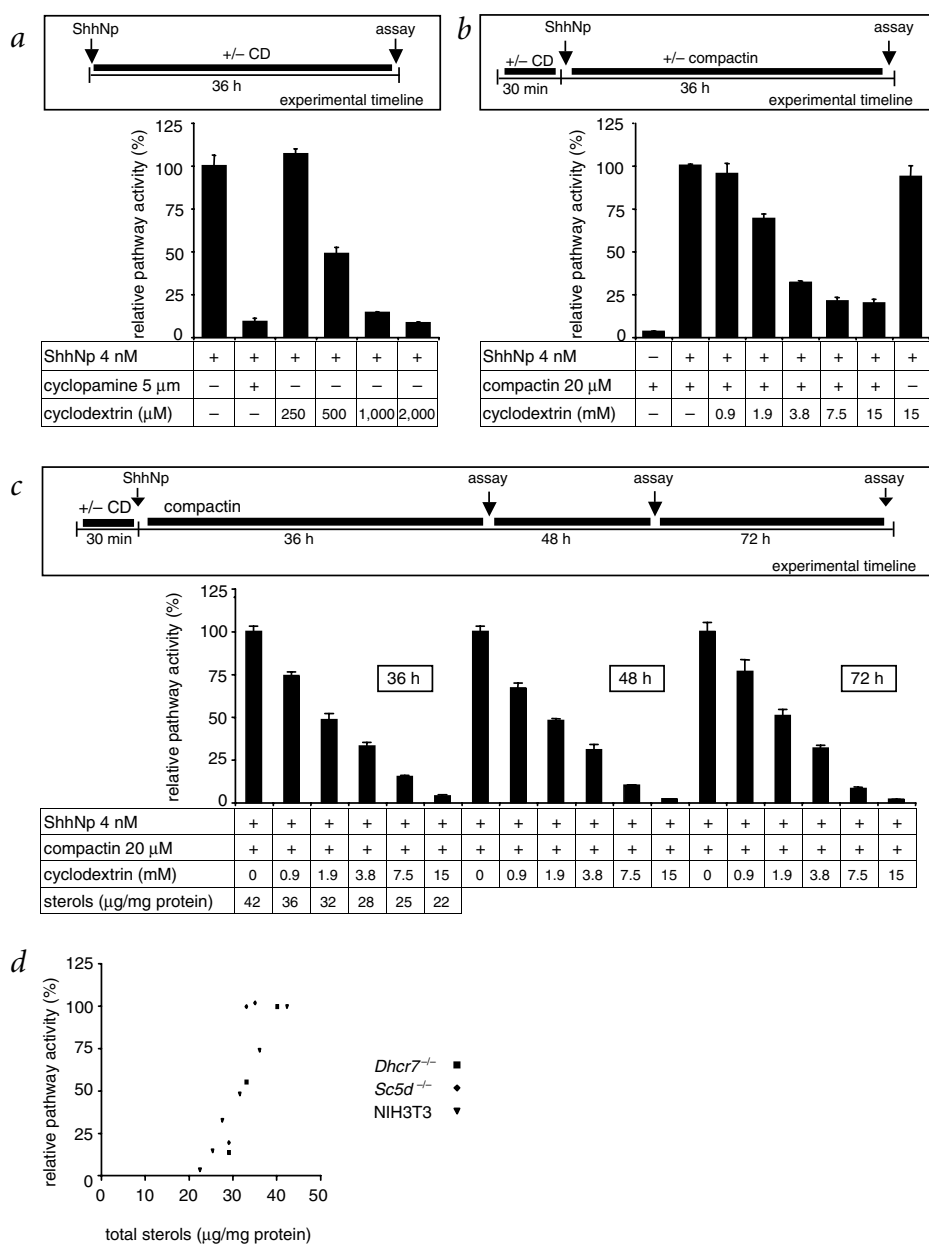
15 mM cyclodextrin, a concentration 30 times higher than the IC50 for continuous cyclodextrin treatment (Fig. 4*b*). ShhNp response was also not affected by continuous exposure to compactin, an inhibitor of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG CoA) reductase that blocks sterol synthesis[13]. The combination of transient cyclodextrin treatment with continuous compactin exposure, however, blocked Shh signal response (Fig. 4*b*). These data suggest that compactin treatment, when combined with transient cyclodextrin treatment, can mimic a genetic defect in sterol biosynthesis to inhibit Shh signal response. The Shh autoprocessing reaction was not affected by these experimental conditions (data not shown).
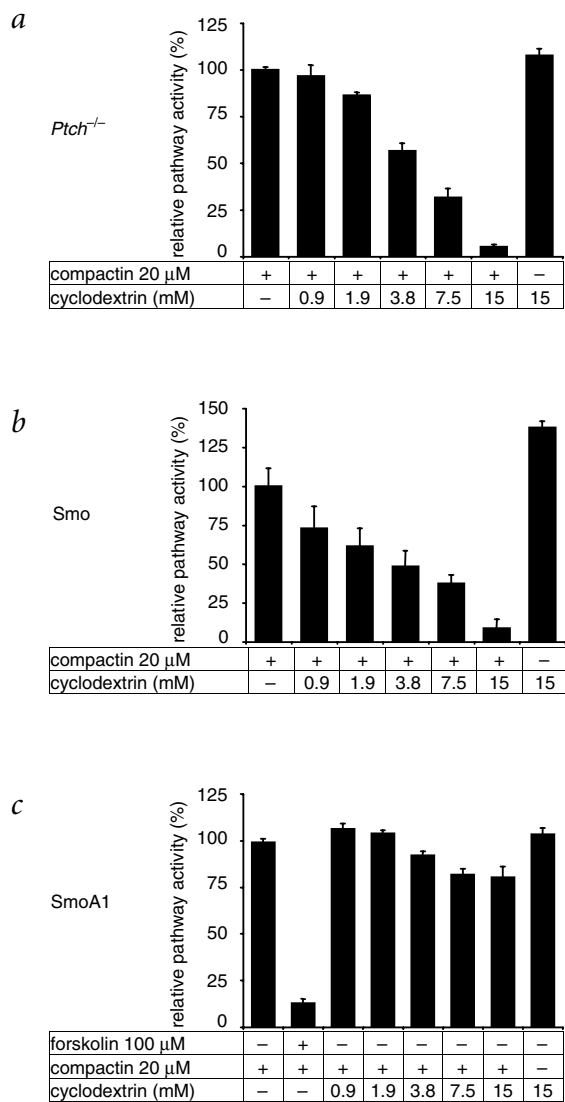
Cyclodextrins form complexes with hydrophobic compounds including proteins and phospholipids in addition to sterols[6]. But the effect of cyclodextrin treatment is probably due to sterol depletion, as transient cyclodextrin treatment only inhibited pathway activity in the presence of sterol biosynthetic mutations or of a statin, and no measurable recovery from the impact of deficits in molecules other than sterols was achieved during prolonged incubation after transient cyclodextrin treatment (Fig. 4*c*). Furthermore, other sterol-specific perturbations, such as treatment with nystatin or filipin[14], also blocked the response of cells to ShhNp protein (data

not shown), and the degree of pathway inhibition in sterol biosynthetic mutant and statin-treated fibroblasts correlated inversely with total sterol levels (Fig. 4*d*). An inhibitory role for 7-dehydrocholesterol, lathosterol and other cholesterol precursors that accumulate in the *Dhcr7* and *Sc5d* mutant cells seems improbable because synthesis of such precursors in statin-treated wild-type cells is blocked. Indeed, the correlation between responsiveness and overall sterol content (Fig. 4*d*) suggests that these precursors may contribute to pathway response, although their effectiveness relative to that of cholesterol is unknown.

The ability to pharmacologically mimic sterol biosynthetic defects affords an opportunity to identify the Hh pathway component most directly affected by sterol perturbations. Patched (Ptch) and Smoothened (Smo) stand out as candidate targets because of their integral membrane structure and essential roles in regulating cellular response to the Hh signal. Genetic and biochemical evidence indicates that Ptch suppresses the activity of Smo and that Hh binding to Ptch releases this suppression, allowing Smo to activate a transcriptional response through the

Fig. 4 Inhibition of Hh signal response by sterol depletion in cells with intact cholesterol biosynthesis. Hh signal response was inhibited either by chronic cyclodextrin treatment (*a*) or by statin exposure after acute cyclodextrin treatment (*b*). *a*, NIH3T3 fibroblasts stably transfected with a Hh-responsive luciferase reporter construct (Shh-LIGHT Z3 cells) were chronically depleted of sterols by the addition of cyclodextrin (CD) to the culture medium for the duration of ShhNp induction (see schematic in *a*). Cyclodextrin treatment inhibited the response to Shh signaling in a dose-dependent manner and to a comparable degree as 5 μM cyclopamine[16]. *b*, Shh signal reception was also blocked in Shh-LIGHT Z3 cells by acute sterol depletion with transient exposure to cyclodextrin before ShhNp induction, followed by the addition of an HMG-CoA reductase inhibitor (compactin) during the period of induction (see schematic in *b*). The combination of transient cyclodextrin treatment followed by inhibition of cholesterol synthesis blocked the response to ShhNp, whereas neither treatment alone did so. *c*, Hh pathway inhibition persisted for 72 h after cyclodextrin treatment. The 3-d recovery period was intended to allow replenishment of non-sterol cellular components depleted by transient cyclodextrin treatment while maintaining sterol depletion with HMG-CoA reductase inhibition by compactin. *d*, Total cellular sterols from parallel cultures of NIH3T3 (*c*, 36 h), *Dhcr7*^{−/−} and *Sc5d*^{−/−} (Fig. 3*b*) MEFs were determined by gas chromatography–mass spectrometry analysis of extracted lipids and plotted against the relative Shh pathway activity. Bars represent one standard error (quadruplicate wells).

a

Ptch⁻/⁻

compactin 20 µM	+	+	+	+	+	+	–
cyclodextrin (mM)	–	0.9	1.9	3.8	7.5	15	15

b

Smo

compactin 20 µM	+	+	+	+	+	+	–
cyclodextrin (mM)	–	0.9	1.9	3.8	7.5	15	15

c

SmoA1

forskolin 100 µM	–	+	–	–	–	–	–	–
compactin 20 µM	+	+	+	+	+	+	+	–
cyclodextrin (mM)	–	–	0.9	1.9	3.8	7.5	15	15

Fig. 5 Sterol depletion inhibited Shh signaling downstream of Ptch at the level of Smo. *a*, The constitutively active Shh pathway in *Ptch⁻/⁻* MEFs (measured as β-galactosidase activity produced from a fusion of *lacZ* to the third codon of Ptch[16]) was blocked by cholesterol depletion. *b*, Shh pathway activation by overexpression of wild-type Smo in NIH3T3 cells was also inhibited by cholesterol depletion. But Shh pathway activity driven by overexpression of oncogenic Smo (*c*; SmoA1, W539L) was resistant to inhibition by cholesterol depletion. Cyclodextrin and compactin treatments for *a–c* are as indicated for Figure 3*b*. NIH3T3 fibroblasts in *b* and *c* were stably transfected with expression constructs for either Smo (*b*) or activated Smo (SmoA1; *c*), a Gli-luciferase reporter and *lacZ* for normalization. Forskolin (100 µM) inhibition of SmoA1-driven pathway activity is illustrated as a positive control[16]. Bars represent one standard error from three (*a*) or six (*b,c*) replicates for each treatment group.

Gli family of transcription factors (Fig. 1*b*). We examined the ability of sterol perturbations to affect the constitutive Hh pathway activity in fibroblasts derived from *Ptch⁻/⁻* embryos[15,16] and found a dose-dependent reduction by transient treatment with increasing concentrations of cyclodextrin in combination with compactin (Fig. 5*a*). Therefore, sterol depletion can block pathway activity independently of Ptch action and may act at a point in the pathway downstream of Ptch.

We next examined the effects of depleting sterols in NIH3T3 cells stably transfected to express wild-type or oncogenic, activated Smo (ref. 16; SmoA1; W539L). We found that sterol depletion could completely block the modest level of pathway activation produced by overexpression of Smo (Fig. 5*b*), but that it scarcely affected pathway activation by SmoA1 (Fig. 5*c*). The susceptibility of wild-type Smo but resistance of a mutant activated form to sterol depletion suggests that Smo may be the site of sterol action. Consistent with this conclusion, sterol deprivation did not affect the activation of the pathway due to expression of Gli1, which acts downstream of Smo (data not shown). The retention of normal Hh pathway activity in cells expressing activated Smo indicates that pathway components downstream of Smo function normally under conditions of sterol depletion.

Previous work has suggested that Smo activity is governed by a balance between active and inactive conformations[16]. The resistance

to cholesterol deprivation of activated Smo suggests that Smo conformation may be the target of cholesterol deprivation. This effect could be mediated either through direct interaction of cholesterol with Smo or through an impact on membrane properties, as reported for the function of other seven-transmembrane-domain proteins, such as the oxytocin or the brain cholecystokinin receptors[17]. One possibility is that sterol depletion could affect a lipid microdomain or raft-mediated process[18] required for Smo activity, although we did not observe a change in Smo fractionation with respect to detergent-resistant membranes on sterol depletion (data not shown). Alternatively, the effects of sterol depletion on Smo activity might be indirectly mediated through an as yet uncharacterized interacting component.

Sterol depletion has been previously reported to affect Shh autoprocessing[10]. This depletion was probably somewhat more severe than that produced by our experimental manipulations. Furthermore, we found that Hh signal response is more sensitive than Hh autoprocessing to inhibition by mutational or pharmacological sterol depletion. We therefore conclude that inhibition of response to Hh protein is a more probable cause of the malformations associated with cholesterol biosynthetic disorders than is inhibition of Hh autoprocessing. Other processes might also be affected by defects in distal cholesterol biosynthesis, as not all of the malformations are necessarily accounted for by impaired Hh signaling. Nevertheless, our findings help explain many developmental malformations associated with a relatively common genetic disorder, SLOS, whose incidence among European Caucasians is 1 in 22,000 (ref. 19). Our findings may also be relevant to other etiologies of abnormal human development, as holoprosencephaly is reported to occur at a frequency of 1 in 250 among aborted fetuses[20]. The surprising connection between cholesterol synthesis and Hh signal response reported here suggests that signaling pathways involved in developmental patterning must be considered as potential targets of any seemingly simple metabolic defect associated with developmental malformations.

Methods

Chick embryos. We applied methyl-β-cyclodextrin (200 µl of a 10% w/v solution in L-15 medium (Sigma and Life Technologies)) to windowed, fertile chick eggs (White Leghorn) after 15 h of incubation. For an average egg volume of 50 ml, the final concentration of cyclodextrin was 375 µM. After 4 d of further incubation, we processed the embryos for scanning electron microscopy. We dissected the neural plate and epidermal ectoderm from stage 9–10 chick embryos, cultured them in collagen and induced them with purified ShhN[21] as described[3]. We added methyl-β-cyclodextrin to the chick explant cultures 4 h after induction with ShhN was initiated.

Analysis of Shh protein biogenesis. We plated MEFs in a 10-cm² dish (Falcon) and transfected them (Fugene 6, Roche) with a full length *Shh* expression construct (pRK5-Shh, 5µg) when the cells were roughly 75% confluent. The next day, we changed the culture medium (Dulbecco's modified Eagle medium with 10% fetal bovine serum) to contain either 0.5% fetal bovine serum, 0.5% lipid-depleted serum or lipid-depleted serum after a

30-min treatment with 3.8 mM methyl-β-cyclodextrin (Sigma). After an additional 24 h in culture, we lysed the MEFs in RIPA buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 1 μg ml^{-1} leupeptin, 1 μg ml aprotinin^{-1}, 0.2 mM phenyl-methylsulfonyl fluoride), immunoprecipitated Shh protein from the cell lysates with a monoclonal antibody that recognizes the N-terminal signaling domain (5E1, Developmental Studies Hybridoma Bank) and immunoblotted them with a polyclonal antibody preparation (JH134).

Shh signaling assays. We carried out Shh signaling assays as described[16] in primary fibroblasts lacking functional Dhcr7, Sc5d or Ptch and in NIH3T3 fibroblasts. *Dhcr7*$^{-/-}$ and *Dhcr7*$^{+/-}$ MEFs were generated from embryonic day–9 mutant mice and *Sc5d*$^{-/-}$ and *Sc5d*$^{+/-}$ MEFs from embryonic day–14.5 mutant mice. We generated a reporter construct with firefly luciferase and Gli (pGL3-8×Gli-luciferase) by cloning eight tandem Gli-binding sites and a lens crystallin promoter from the 8×Gli-BS Luc construct[22] into the pGL3-Basic vector (Promega). We determined relative Hh pathway activity in *Dhcr7*$^{-/-}$, *Dhcr7*$^{+/-}$, *Sc5d*$^{-/-}$ and *Sc5d*$^{+/-}$ MEFs from the expression of transiently transfected pGL3-8×Gli-luciferase and control *Renilla* luciferase (pRL-SV40; Promega) vectors. In *Ptch*$^{-/-}$ MEFs, we normalized β-galactosidase activity expressed under the control of the *Ptch* promoter for protein levels to determine relative Hh pathway activity. In the NIH3T3 cell clone Shh-LIGHT Z3, we normalized 8×Gli-BS luciferase activity for β-galactosidase activity from stably transfected vectors (8×Gli-BS Luc[22] and pIZ-lacZ). We established clonal sublines of Shh-LIGHT Z3 by cotransfecting either Smo tagged with Myc epitope or SmoA1 with vector encoding G418 resistance (pGT; Inviogen). All transfections were done with Fugene 6. We carried out cyclodextrin treatments for 30 min with methyl-β-cyclodextrin (Sigma) dissolved in Dulbecco's modified Eagle medium, preceded and followed by two washes with phosphate-buffered saline. Fetal bovine serum was depleted of lipids as described[23]. We purchased compactin as an active sodium salt form of mevastatin (Biomol) and dissolved it in dimethylsulfoxide.

Sterol analysis. We extracted neutral sterols and analyzed them as described[24] from replicate wells of MEFs cultured in parallel with those used for Hh signaling assays.

Competing interests statement

The authors declare that they have no competing financial interests.

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