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# Negative regulation of Hedgehog signaling by the cholesterologenic enzyme 7-dehydrocholesterol reductase

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Cholesterol regulates Hedgehog (Hh) signaling during early vertebrate development. Smith-Lemli-Opitz syndrome (SLOS) is caused by defects in 7-dehydrocholesterol reductase (DHCR7), an enzyme catalyzing the final step of cholesterol biosynthesis. Many developmental malformations attributed to SLOS occur in tissues and organs where Hh signaling is required for development, but the precise role of DHCR7 deficiency in this disease remains murky. We report that *DHCR7* and *Sonic Hedgehog* (*Shh*) are co-expressed during midline development in *Xenopus* embryos. DHCR7 has previously been implicated to function as a positive regulator of Hh signaling that acts to regulate the cholesterol adduction of Hh ligand or to affect Hh signaling in the responding cell. We present gain- and loss-of-function analyses suggesting that DHCR7 functions as a negative regulator of Hh signaling at the level or downstream of Smoothened (*Smo*) and affects intracellular Hh signaling. Our analysis also raises the possibility that the human condition SLOS is caused not only by disruption of the enzymatic role of DHCR7 as a reductase in cholesterol biosynthesis, but may also involve defects in DHCR7 resulting in derepression of *Shh* signaling.

**KEY WORDS:** *Xenopus*, Midline, Cholesterol, Floor plate, Smith-Lemli-Opitz syndrome (SLOS), Sonic hedgehog

## INTRODUCTION

The members of the Hedgehog (Hh) family are involved in a wide variety of processes, including establishment of midline structures, somite development and digit formation. After translation, Hh proproteins autoproteolytically cleave their ~45 kDa precursor to generate two mature proteins, a N-terminal 'signaling' ligand and a C-terminal cleavage product (Lee et al., 1994). Autoproteolysis occurs concomitantly with the covalent attachment of cholesterol to the C terminus of the Hh ligand (Porter et al., 1996). Hh ligands are also palmitoylated at a highly-conserved Cys residue (Chamoun et al., 2001; Pepinsky et al., 1998) and these lipid modifications lead to production of a soluble multimeric protein complex that is essential to ensure effective potency and range for Hh signaling (Chen et al., 2004; Lewis et al., 2001; Zeng et al., 2001).

Hh proteins bind to their receptor Patched (Ptc), a 12-pass transmembrane protein (Ingham and McMahon, 2001; Lum and Beachy, 2004). This binding relieves the inhibitory effect of Ptc on the seven-pass transmembrane protein Smoothened (*Smo*), thereby transducing the Hh signal to the interior of the cell. Once *Smo* activity is triggered, *Gli/Ci* transcription factors are activated that induce downstream target genes. At present, the details of the Hh signaling cascade between the relief of *Smo* inhibition and the activation of *Gli/Ci* are not well understood. However, in vertebrates, the activity of the *Gli* protein is negatively regulated by protein kinase A (PKA) (Hammerschmidt et al., 1996).

Involvement of cholesterol in Hh signaling was further suggested by sensitivity of the response to perturbation of cholesterol homeostasis. A number of reagents known to inhibit cholesterol

transport, such as cyclopamine, jervine, progesterone and U1866A, block Hh signaling in chick neural plate explants (Cooper et al., 1998). Among these agents, cyclopamine has also been shown to specifically bind to *Smo* (Chen et al., 2002). Additionally, AY-9944, an inhibitor of cholesterol biosynthesis, also blocks *Shh* signaling in vitro (Cooper et al., 1998; Incardona et al., 1998). Together, these results point to the importance of cholesterol regulation during embryogenesis, with the implication that disturbance of this process could result in the types of developmental defects known to be associated with abnormal Hh signaling.

Aberrant Hh signaling underlies a number of human diseases and congenital defects, including basal cell carcinoma, polydactyly and holoprosencephaly (HPE) (Ingham and McMahon, 2001). HPE is a patterning defect characterized by incomplete separation of the two halves of the forebrain at the midline. Similarly, HPE is also observed in the offspring of pregnant rats treated with AY-9944, which inhibits cholesterol biosynthesis by inhibiting the enzymatic activity of 7-dehydrocholesterol reductase (DHCR7) (Roux and Aubry, 1966). DHCR7 is a nine-pass membrane protein that catalyzes the final cholesterologenic step in the endoplasmic reticulum by reducing 7-dehydrocholesterol (7-DHC) to cholesterol (Fitzky et al., 1998; Wassif et al., 1998). Milder manifestations of HPE have been observed in ~5% of individuals with Smith-Lemli-Opitz Syndrome (SLOS), which is an autosomal recessive human disorder that results from mutations in *DHCR7* (Kelley et al., 1996). In addition to midline defects, individuals with SLOS also suffer from malformations in the limbs, heart, kidneys, pancreas and genitals (Kelley and Hennekam, 2000). Explanations of how blockade of the cholesterol biosynthetic pathway leads to these malformations include (1) low levels of cholesterol and/or (2) the accumulation of sterol precursors in tissues, either of which are proposed, in turn, to affect the development of various tissues and organs during embryogenesis.

Several groups have shown that cholesterol deficiency can interfere with normal Hh signaling, and have proposed that this deficiency is the root cause of some of the developmental abnormalities seen in individuals with SLOS (Cooper et al., 2003; Guy, 2000). However, direct evidence demonstrating that impaired

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DHCR7 function leads to cholesterol deficiency that, in turn, blocks Hh signaling is notably lacking. Because cholesterol can be supplied to early embryos maternally, it is difficult to assess the requirement of DHCR7 activity during early development. Additionally, although some correlations exist between decreased levels of plasma cholesterol and increased levels in the clinical severity of SLOS, there are also inconsistencies between the acuteness of the SLOS phenotype and the plasma levels of cholesterol and its precursor 7-DHC (Witsch-Baumgartner et al., 2000). Finally, neither treatment of cells with AY-9944 nor deletion of DHCR7 in mouse embryonic fibroblasts has proven effective in blocking sterol adduction of Shh (Cooper et al., 1998; Cooper et al., 2003).

In order to uncover the function of DHCR7 during mouse embryogenesis, two DHCR7 mutants have been generated in this animal model. In the first, exon 8 was removed, generating the mutation DHCR7<sup>ΔEX8</sup>, which resembles DHCR7<sup>IVS8-1G>C</sup>, a mutation frequently encountered in individuals with SLOS (Fitzky et al., 2001). Another mutant, DHCR7<sup>Δ3-5</sup>, was produced by deleting exons 3, 4 and 5, thus eliminating most of the transmembrane-spanning region (Wassif et al., 2001). Both of these alterations inactivate the reductase activity of DHCR7, resulting in impaired conversion of 7-DHC to cholesterol. Further characterization of the mutant mice also revealed an overall accumulation of sterol precursors and reduction in tissue cholesterol levels; importantly, similar changes have been observed in human SLOS. However, neither mutant displayed morphological defects in the developing central nervous system or limbs, tissues where Shh is known to play crucial roles. Therefore, the precise relationships between DHCR7 activity, Hh signaling and SLOS need to be clarified.

We identified *Xenopus* DHCR7 in a microarray-based screen to uncover genes involved in chordamesodermal specification during gastrulation. To characterize the role of DHCR7 during early vertebrate development, we have performed both loss- and gain-of-function analyses in early *Xenopus* embryos. We report that DHCR7 functions contrary to previously held notions. DHCR7 acts as a negative regulator of the response to Shh signaling during early embryogenesis, and its inhibitory effects appear to impinge on the Hh signaling pathway at the level of smoothed (Smo).

## MATERIALS AND METHODS

### Embryo manipulations

*Xenopus* embryos were fertilized in vitro and processed as described (Blitz and Cho, 1995). Embryos were microinjected and maintained with or without 5–100 μM AY-9944. Embryos were treated with cyclopamine continuously from the blastula stage (Perron et al., 2003). Capped mRNAs were prepared using an Ambion mMessage Machine kit. Animal cap explants were dissected and cultured as described (Cho et al., 1991). For luciferase reporter assays, *Gli*-luciferase reporter (0.2 ng) (Sasaki et al., 1997) was microinjected into two-cell stage embryos alone or with combinations of *Chordin*, *Shh-N* and *DHCR7* mRNAs. Animal caps were dissected (stage 8), cultured until control embryos reached stages 12–18 and subjected to luciferase assays. All reporter gene experiments were repeated a minimum of three times. Animal cap conjugation experiments were conducted by recombining two animal caps expressing indicated mRNAs and a reporter gene. The conjugates were cultured and assayed at indicated stages.

Whole-mount in situ hybridization (Harland, 1991) was performed using BM purple (Roche) as substrate. In situ probes for *Shh*, *DHCR7*, *Pax6*, *Pax2*, *HNF3β/FoxA2*, *Ptc1* and *Gli1* were prepared by digesting the DNAs with *EcoRI* and transcribing with T7 RNA polymerase.

### Microarray analysis

Total RNA was isolated from microdissected notochordal and presomitic mesodermal tissue fragments from early neurulae. Fluorescent labeled probes were generated based on a T7-based RNA amplification method

(Wang et al., 2000). Four micrograms of amplified RNA were reverse transcribed in the presence of amine-modified random hexamers, and subsequently fluorescently labeled using a Cy3- and Cy5 labeling kit (Amersham). Generation of microarray slides, slide hybridization and scanning were as described previously (Shin et al., 2005). A GenePix Pro image acquisition program and Expressionist software (GeneData) were used for array data analyses.

### Plasmids

*Xenopus* DHCR7 was subcloned into pCS2+ (Turner and Weintraub, 1994) to generate DHCR7 mRNA transcripts. A DHCR7 rescue construct was created by inserting a PCR fragment between the *EcoRI* and *Ascl* sites of pCS2 AT+ (Tsuji and Hashimoto, 2005). Primers used were DHCR7<sup>wt-F</sup> (5'-GGAATTCGACACCATGGGAGAGCGGAGAAGAG-3') and DHCR7<sup>wt-R</sup> (5'-GGCGCGCCAAAGGTGAGGCGGTAAAA-CA-3'). DHCR7 mutants were generated by PCR-based mutagenesis using the full-length DHCR7 cDNA as a template. The combinations of the primers used to generate DHCR7 mutants were: DHCR7<sup>wt-F</sup>, DHCR7<sup>wt-R</sup>, DHCR7<sup>R350W-F</sup> (5'-TACATCTTCTGGATGACCAATCAC-3') and DHCR7<sup>R350W-R</sup> (5'-GCTGACACGTAAAAACATTCGATG-3') for DHCR7<sup>R350W</sup>; DHCR7<sup>wt-F</sup> and DHCR7<sup>IVS8-1G>C-R</sup> (5'-GGCGCGCCCTACTACTGTAGGGTATAGAGGTAGGG-3') for DHCR7<sup>IVS8-1G>C</sup>; DHCR7<sup>wt-F</sup> and DHCR7<sup>W149X-R</sup> (5'-GGCGCGCCGACCCATT-3') for DHCR7<sup>W149X</sup>; and DHCR7<sup>wt-F</sup>, DHCR7<sup>wt-R</sup>, DHCR7<sup>ΔN1-F</sup> (5'-CTGTGGTGCGCCAATCTC-3') and DHCR7<sup>ΔN2-F</sup> (5'-ATGGGAGAGCGGAGAAGAGCGAATCTGTGGTGCGCCAATCTC-3') for DHCR7<sup>ΔN</sup>. All DHCR7 templates were digested with *NotI* and transcribed using SP6 polymerase. Full-length *Shh* and *Shh-N* mRNA were synthesized with T7 polymerase using *EcoRI*-linearized XshhTST7 or Xshh TST7-N templates (Ekker et al., 1995a). *NotI*-linearized pCS2+dnPKA (Ungar and Moon, 1996) and pCS2+XSmoM2-FLAG (Koebernick et al., 2003) templates were transcribed with SP6 polymerase. *chordin* RNA was synthesized as described (Blitz et al., 2000).

### Morpholinos and RT-PCR

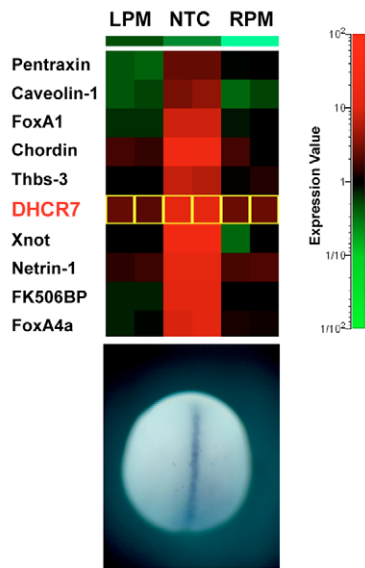
Morpholino oligos (GeneTools) used to inhibit DHCR7 function were DHCR7a-MO (5'-TCTGCTCTTTGTGTTCTGCTTATCT-3') and DHCR7b-MO (5'-GTCCCCAGCAGCTCTCCCCATGTAG-3'). An equimolar mixture of these MOs is referred as DHCR7-MO. Control MO (5'-CCTCTTACCTCAGTTACAATTTATA-3') has no observable effect on *Xenopus* development.

RT-PCR primers used to amplify *histone H4* were as described previously (Blitz and Cho, 1995). DHCR7 primers were 5'-AGACCTCTTCCGACGCACTA-3' and 5'-CCAGGGACCCCATTAATCT-3'; *Shh* primers were 5'-CAGCGACTTCCTCATGTTCA-3' and 5'-GGCAGTTAGAGGCGCATAAG-3'; *Gli1* primers were 5'-GAGCTAGTGACCCTGCAAG-3' and 5'-CATCGGGACCTGCTGTTCC-3'; *Ptc1* primers were 5'-TGCAAGGACTGCAAGATACG-3' and 5'-ATCCACCAACCGCTGTTAG-3'; *FoxA2* primers were 5'-CTTCCAGCACATTCTGT-3' and 5'-ATGGGGACCCGTAAGTACTAGA-3'.

## RESULTS

### Isolation of *Xenopus* DHCR7 using cDNA microarrays

A large-scale cDNA microarray screen was conducted to identify genes specifically expressed in *Xenopus* notochord. RNAs isolated from notochord, and left and right presomitic mesoderm of neurula stage embryos (stage 14–15) were hybridized to cDNA microarrays consisting of 21,000 clones representing ~8500 transcripts (Shin et al., 2005). Hierarchical clustering analysis identified several genes, including *Pentraxin*, *Caveolin-1*, *FoxA1*, *Chordin*, *Xnot*, *FK506BP*, *FoxA4A* and *DHCR7*, which are differentially expressed in the developing notochord (Fig. 1). Among these, DHCR7 (7-dehydrocholesterol reductase, EC 1.3.1.21) was particularly interesting as this enzyme has a presumed role in Hh signaling. Whole-mount in situ hybridization confirmed the expression of *DHCR7* in the midline of early neurula stage embryos (Fig. 1).



**Fig. 1. Expression of *DHCR7* in developing notochord.**

(Top) Hierarchical clustering analysis of microarray data. Each column represents a single hybridization and each row a single clone. The colors ranging from red to green correspond to increased and decreased gene expression. LPM, left presomitic mesoderm; NTC, notochord; RPM, right presomitic mesoderm. (Bottom) Whole-mount in situ hybridization revealing the expression of *DHCR7* in the midline of a *Xenopus* neurula stage embryo.

Two related *DHCR7* cDNAs were identified from the *Xenopus* EST (<http://Xenopus.nibb.ac.jp/>) and TIGR databases (see Fig. S1 in the supplementary material). The predicted amino acid sequences of these *Xenopus DHCR7* cDNAs were 95% identical, and the activities of these gene products appeared to be identical in all the assays performed (T.K., T.H. and K.W.Y.C., unpublished). Therefore, we conclude that these similar cDNAs represent duplicated forms of *DHCR7* arising from the pseudotetraploidy of *Xenopus laevis*, and are hence designated as *DHCR7a* and *DHCR7b*. The predicted *Xenopus DHCR7* protein consists of 473 amino acids and the percentage of identity at the amino acid sequence level against its mouse, human, rat and zebrafish counterparts is 77% (see Fig. S1 in the supplementary material). *Xenopus DHCR7* possesses a conserved 180 amino acid sterol-sensing domain (SSD) (residues 179-360) that is 86% identical to that of human *DHCR7* (residues 181-362). The significance of the possession of an SSD in *DHCR7* that is related to SSDs in other proteins is currently unknown.

### DHCR7 expression in *Xenopus* embryos

As *DHCR7* has been implicated in Hh signaling, we looked for clues to its relationship with Shh during *Xenopus* embryogenesis. Temporally, *DHCR7* transcripts are first detected at the blastula stage (stage 9). As *Shh* is initially transcribed at the early gastrula stage (stage 10) (Ekker et al., 1995a), *DHCR7* expression precedes that of *Shh*. Both *DHCR7* and *Shh* transcripts continue to be expressed during early embryogenesis at least until tadpole stages (Fig. 2A).

Whole-mount in situ hybridization revealed that shortly after the onset of gastrulation, *DHCR7* is expressed in the organizer and extends broadly along the marginal zone (Fig. 2B, part a), whereas *Shh* is first detected weakly in the organizer with expression restricted to the dorsal midline of the gastrula (Fig. 2B, part e). As neurulation proceeds, expression of both *DHCR7* and *Shh* mRNA

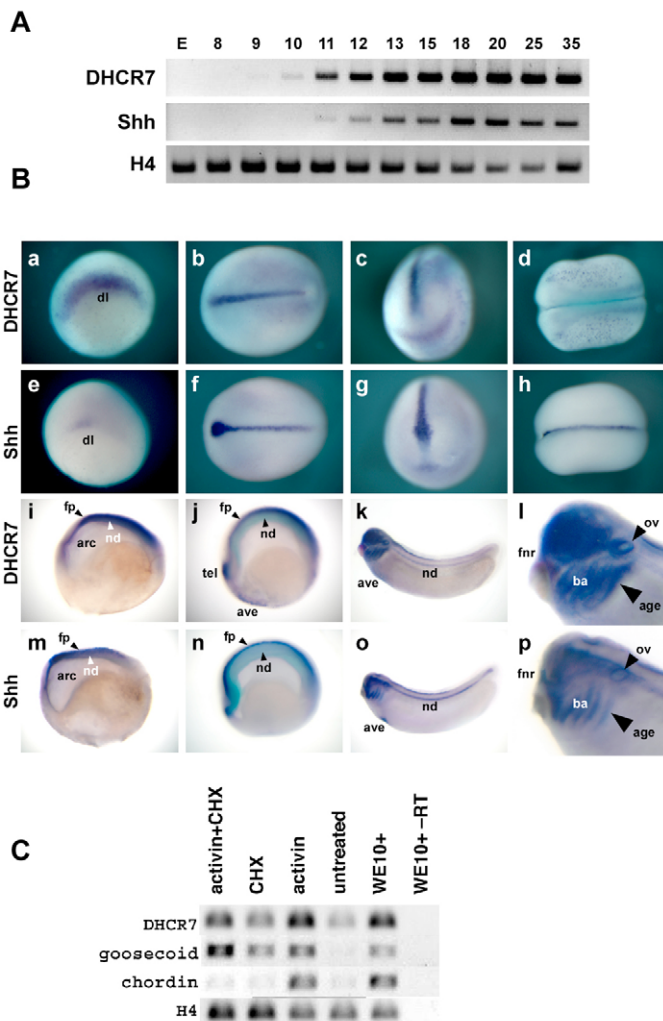
extend along the embryonic dorsal midline (Fig. 2B, parts b, f). By late neural stages (stage 20), *DHCR7* is expressed in the presumptive telencephalon and anterior ventral endomesoderm (ave) with weak expression in the neural plate, epidermis and notochord (Fig. 2B, parts c,d,j), while *Shh* is expressed in the floor plate, prechordal plate and notochord (Fig. 2B, parts g,h,n). At the tailbud stage, both *DHCR7* and *Shh* are similarly expressed in the notochord, ventral central nervous system, floor plate, branchial arches, frontonasal region, and presumptive anterior gut endoderm, and in a very limited region of the ave (Fig. 2B, parts k,l,o,p). In conclusion, although there are differences in the details of their expression, the overall patterns are similar for *Shh* and *DHCR7*, allowing for the existence of a functional link between their activities.

We then used assays in animal cap (ectodermal) tissue explants to examine how the expression of *DHCR7* is controlled. Overexpression of Shh, BMP or Wnt in animal caps did not induce *DHCR7* (data not shown). However, treatment with activin protein, a member of the TGF $\beta$  superfamily, induced *DHCR7* expression, and this induction was direct as *DHCR7* was upregulated even in the presence of the protein synthesis inhibitor cycloheximide (Fig. 2C). These results implicate a potential role for TGF $\beta$  signaling in the regulation of the Shh signal cascade during early *Xenopus* embryogenesis.

### Gain-of-function analysis suggests *DHCR7* is an inhibitor of Shh signaling

Sterol modification of Shh appears to be obligatory for proper Shh signaling in the processing of the precursor to produce the Hh ligand, suggesting that *DHCR7* is an important enzyme positively influencing the activity of Shh. To explore the function of *DHCR7* during early embryogenesis, we examined the effects of *DHCR7* overexpression. Embryos overexpressing *DHCR7* mRNA appeared to develop relatively normally until tailbud stages (data not shown). At the swimming tadpole stage, however, embryos overexpressing *DHCR7* developed ectopic pigmentation in the optic stalk (Fig. 3A; 63%,  $n=46$ ). This is a surprising observation as this phenotype resembles that reported in mice deficient in Shh in retinal ganglion cells (Dakubo et al., 2003) and suggests that *DHCR7* may function as a negative regulator of Hh signaling. We therefore examined whether *DHCR7* is capable of reversing phenotypes induced by Shh overexpression. To accomplish this, we injected *Shh* mRNA alone or together with *DHCR7* mRNA, and examined the expression of genes influenced by Shh. Previous work has shown that enhancement of Shh signaling promotes the expression of *Pax2* at the expense of *Pax6* expression in optic vesicles (Hammerschmidt et al., 1996; Macdonald et al., 1995; Ungar and Moon, 1996). *Pax6* expression, which is inhibited by Shh, is rescued by co-injection of *DHCR7* mRNA (Fig. 3B). Likewise, the expression of *Pax2*, *Gli1* and *Ptc1*, which is stimulated by Shh overexpression, can be rescued by co-expression of *DHCR7* mRNA.

Next, we examined the behavior of *DHCR7* in a more defined system: animal cap assays. When the BMP antagonist Chordin is expressed in animal cap ectodermal explants, the caps are converted to a neural fate (Sasai et al., 1994). Chordin expression in the presence of Shh effectively induces Shh targets such as *Gli1* (Tsuda et al., 2002) (Fig. 3C, lane 2). However, expression of *DHCR7* significantly reduced the expression of *Gli1*, *Ptc1* and *FoxA2* (Fig. 3C, lane 3) consistent with an inhibitory role for *DHCR7* in Shh signaling. To examine whether this inhibitory behavior of *DHCR7* is independent of the cholesterol adduction of Shh, we examined the effect of *DHCR7* on a Shh-N-mediated Hh response. Shh-N, a N-terminal Shh ligand produced from recombinant Shh mRNA lacking



**Fig. 2. Developmental expression profiles of *DHCR7* and *Shh*.** (A) RT-PCR analysis of *DHCR7* and *Shh* expression during *Xenopus* development. *H4*, histone *H4*. (B) Whole-mount in situ hybridization of *DHCR7* and *Shh* at gastrula (a,e), neurula (b-d,f-h,i,j,m,n), tadpole (k,l,o,p) stages. (a,b,d,e,f,h) Dorsal views. (c,g) Anterior views. (i-p) Lateral views with anterior towards the left and dorsal upwards. dl, dorsal lip; fp, floor plate; arc, archenteron; tel, telencephalon; ave, anterior ventral endoderm; ba, branchial arches; fnr, frontonasal region; age, presumptive anterior gut endoderm; nd, notochord; ov, otic vesicle. (C) Induction of *DHCR7* by activin in animal cap explants. CHX, cycloheximide; WE, whole embryos.

the C-terminal domain, does not require cholesterol adduction for its activity, but is as active as full-length Shh construct in overexpression studies (Ekker et al., 1995a; Lai et al., 1995). *DHCR7* efficiently blocked *Shh-N*-induced *Gli1*, *Ptc1* and *FoxA2* expression in both animal caps (Fig. 3C, lanes 4 and 5) and embryos (data not shown), suggesting that the role of *DHCR7* in antagonizing Shh signaling does not occur at the level of cholesterol modification of Shh ligand.

To determine whether *DHCR7* impinges on Hh signaling, we used a Gli reporter gene (8×3'-Gli-BS) (Sasaki et al., 1997), which was shown to respond to Hh signaling directly. The Gli reporter is activated in animal caps expressing Chordin and *Shh-N*, and this activation is blocked in the presence of *DHCR7* (Fig. 3D). This Gli reporter activation is due to Hh signaling, as mGli-BS (a reporter

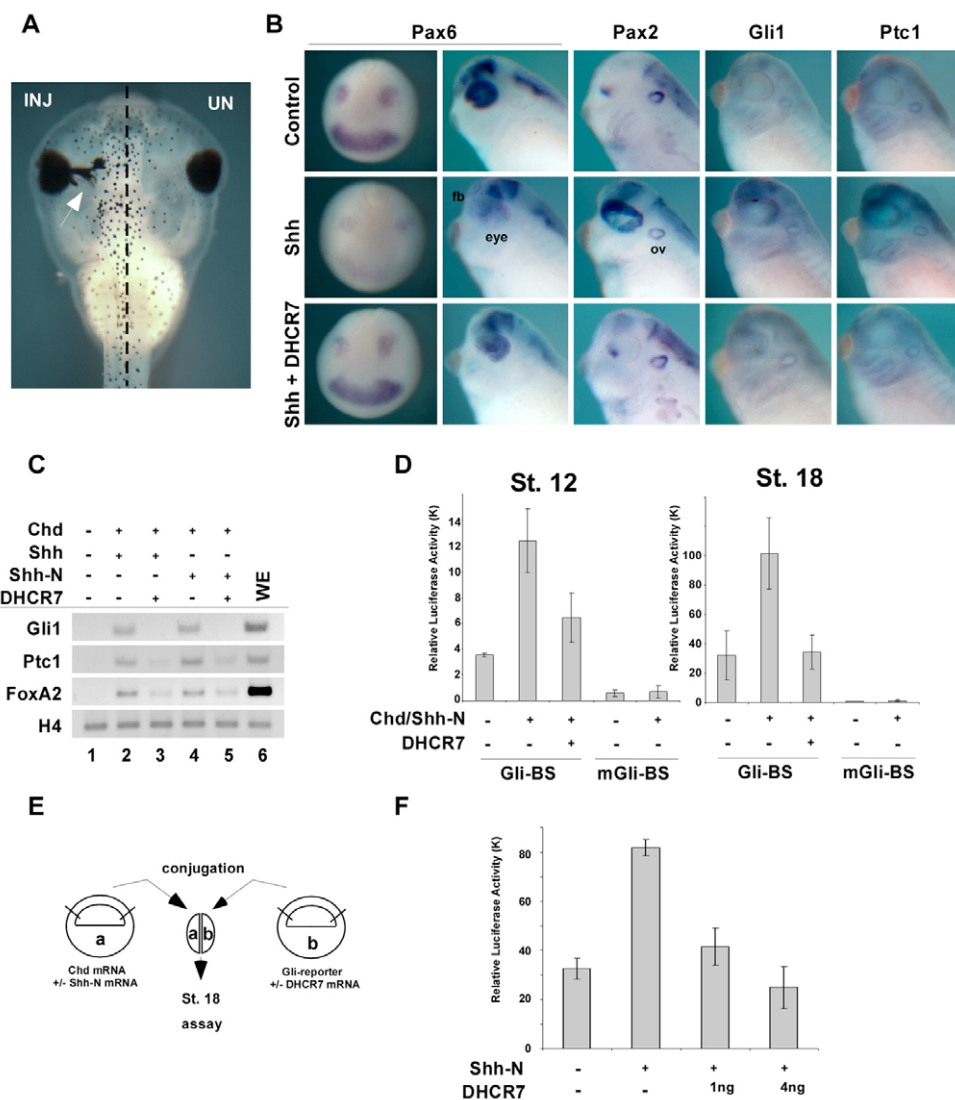
gene with mutations in Gli-binding sites) was not induced by *Shh-N*. We observed the effect of *Shh-N* on Gli transcription as early as stage 12 embryos.

We also wished to determine whether *DHCR7* inhibits Hh signaling by blocking responding cells from reacting to the signal. To address this issue, we used an animal cap conjugation assay (Fig. 3E). Animal caps expressing Chordin with or without *Shh-N* mRNA were used to provide the Shh protein to conjugated animal caps injected with Gli-reporter in the presence or absence of *DHCR7*. The recombined caps were then incubated and subjected to a luciferase assay. We found that induction of Gli-BS reporter was effectively blocked by *DHCR7* (Fig. 3F), indicating that *DHCR7* inhibits signaling in Hh responding cells and not at the level of production of the ligand.

### Loss-of-function analysis: *DHCR7* is an inhibitor of Shh signaling

Antisense morpholino oligonucleotides (MOs) designed to recognize both *DHCR7a* and *DHCR7b* were injected into developing *Xenopus* embryos. *DHCR7*-MO-injected embryos had developed small eyes with reduced retinas and pigmented epithelia in the ventral half of the eye (Fig. 4B,E; 90%, *n*=48). This phenotype was efficiently rescued by co-expression of *DHCR7* mRNA lacking the MO-binding sequence (Fig. 4C,F; 78%, *n*=40). Combined injection of MOs designed against both *DHCR7a* and *DHCR7b* gave stronger phenotypes than injection of the individual MOs alone (data not shown). Interestingly, this *DHCR7* phenotype is similar to loss-of-function phenotypes for the known Hh antagonists Rab23 and FKBP8, which result from an inappropriate activation of Shh signaling within the optic vesicle (Bulgakov et al., 2004; Eggenschwiler et al., 2001). The similarities between these phenotypes are consistent with the notion that *DHCR7* functions as a negative, rather than a positive, regulator of Shh signaling. To address whether the defects in optic vesicle patterning are due to the effect of *DHCR7* on Shh signaling, rather than by other indirect effects, we injected *DHCR7*-MO into *Xenopus* embryos and examined the expression patterns of marker genes known to be regulated by Shh signaling. Expression of *Pax6* was reduced in *DHCR7* MO-injected embryos (compare Fig. 4G with H; 70%, *n*=30), accompanied by slightly expanded expression of *Pax2* in the ventral eye (Fig. 4K,L; 60%, *n*=28). *Gli1* was also broadly upregulated in MO-injected embryos (Fig. 4O,P; 58%, *n*=22). Thus, the *DHCR7* loss-of-function effects resemble the phenotypes observed after *Shh* overexpression or PKA inhibition in *Xenopus* and zebrafish (Ekker et al., 1995b; Macdonald et al., 1995; Perron et al., 2003).

As loss-of-function suggests that *DHCR7* is an inhibitor of Shh signaling, we examined whether loss of *DHCR7* enhances responsiveness towards Hh signaling. Therefore, we co-injected *DHCR7*-MO with low doses of *Shh-N* mRNA that, alone, only weakly alter the expression of *Pax2*, *Pax6* and *Gli1* expression. Stimulation of Hh signaling by injecting 50 pg of *Shh-N* mRNA alone resulted in a reduction of *Pax6* (Fig. 4I; 80%, *n*=16) with concomitant expansion of *Pax2* (Fig. 4M; 80%, *n*=22) in the ventral half of the eye. Importantly, embryos co-injected with *DHCR7*-MO and *Shh-N* mRNA showed complete suppression of *Pax6* expression (Fig. 4J; 80%, *n*=16) and expansion of *Pax2* in the entire optic vesicle (Fig. 4N; 80%, *n*=16). These effects were similar to those of embryos receiving high Hh signaling (Lupo et al., 2005), suggesting that the loss of *DHCR7* enhances the cellular response towards Hh signaling during optic vesicle patterning.



**Fig. 3. Inhibition of Shh signaling by DHCR7.** (A) *DHCR7* mRNA (2 ng) was microinjected unilaterally into one blastomere of the two-cell stage embryo. (B) Two-cell stage embryos were injected with 250 pg of *Xenopus Shh* mRNA (middle row), or together with 500 pg of *DHCR7* mRNA (bottom row; Shh + *DHCR7*). Embryos were subjected to whole mount in situ hybridization at stage 35 (tailbud) using indicated probes. When *Shh* mRNA was co-injected with *DHCR7* mRNA, marker gene expression was restored to the wild-type level (top row). The rescue efficiency was: *Pax6*, 87% ( $n=46$ ); *Pax2*, 90% ( $n=48$ ); *Gli1*, 91% ( $n=34$ ); *Patched1*, 93% ( $n=28$ ). (C) RT-PCR analysis of RNA isolated from animal caps injected with *Chordin* (50 pg) plus *Shh* (100 pg) (lane 2), *Chordin* plus *Shh* with 1 ng of *DHCR7* (lane 3), *Chordin* plus 100 pg of *Shh-N* (lane 4) and *Chordin* plus *Shh-N* with 1 ng of *DHCR7* (lane 7) mRNA. Uninjected stage 28 equivalent animal cap control (lane 1). (D) Gli-reporter assays using animal cap assay. (E) Schema of a conjugation experiment. (F) Gli-reporter gene assay using animal cap conjugates.

**DHCR7 functions as a Shh inhibitor in neural tube patterning**

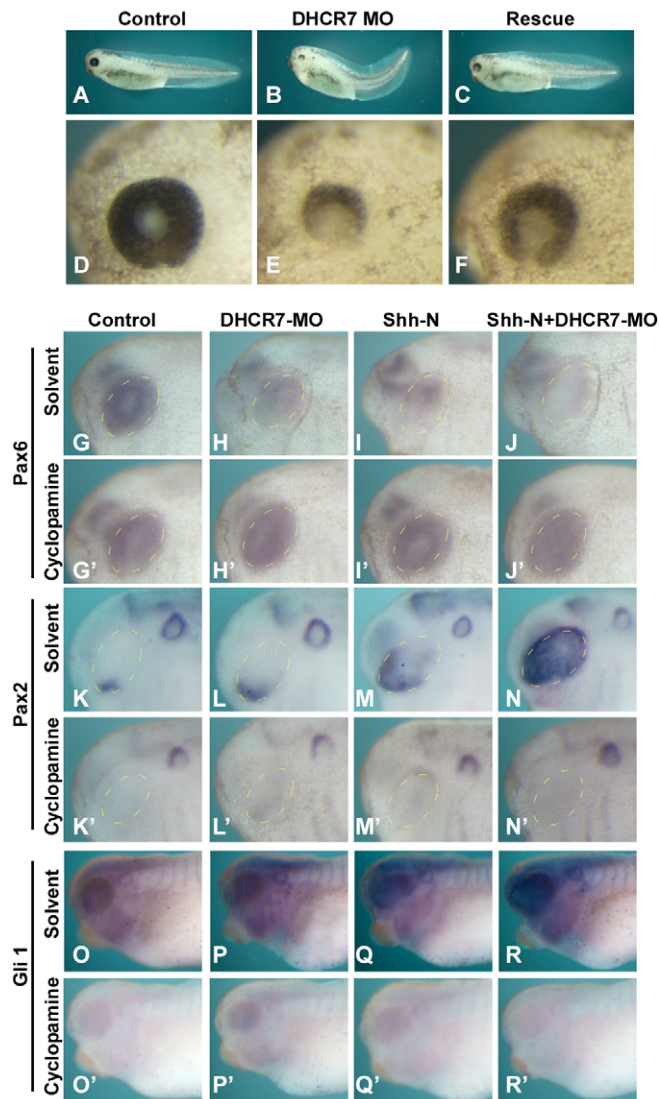
Shh plays an important role in patterning the ventral neural tube. Therefore, we wished to know whether DHCR7 also acts as a negative regulator of Shh in that region. Whole-mount in situ hybridization analysis of *DHCR7* MO-injected embryos showed significant increases in the expression levels of both *Hnf3β/FoxA2* (Fig. 5A,B; 78%,  $n=28$ ) and *Shh* (Fig. 5D,E,J,K,M,N; 92%,  $n=26$ ), which are the markers for the midline. To test further the effect of DHCR7 MO on ventral neural patterning, we then performed unilateral injection of DHCR7 MO (Fig. 6A). Expression of ventral neural tube markers such as *Shh*, *FoxA2* and *Nkx2.2* expanded dorsally in MO-injected side (Fig. 6). Expression of *Dbx1*, which marks the intermediate region of the spinal cord, is also narrowed (Fig. 6H,I), in agreement with the previous finding that high levels of Shh signaling repress *Dbx1* expression (Pierani et al., 1999; Briscoe et al., 2000). All these phenotypes were effectively rescued by overexpression of *DHCR7* mRNA (Fig. 5C,F,I,L,O; Fig. 6D,G,J). These phenotypes are consistent with the notion that DHCR7 loss-of-function causes partial ventralization of the neural tube owing to upregulation of Shh signaling.

**De novo cholesterol synthesis is not essential for Shh signaling during *Xenopus* early embryogenesis**

We next examined how DHCR7 might function together with an inhibitor of cholesterol biosynthesis, in particular AY-9944, a pharmacological inhibitor of the enzymatic activity of DHCR7 (Roux and Aubry, 1966). When *Xenopus* embryos were continuously treated with AY-9944 (up to 100 μM) from the early gastrula stage onwards, no obvious morphological defects were noted (compare Fig. 7C with 7D). Similarly, when animal cap explants were treated with 10 μM AY-9944, a fivefold higher concentration than that necessary to inhibit completely *FoxA2* induction by Shh-N in chick neural plate explants (Cooper et al., 1998), induction of the Gli-reporter by Shh-N was largely unaffected (Fig. 7B). These results suggest that inactivation of the enzymatic activity of DHCR7 by AY-9944 alone may not be sufficient to inhibit Shh signaling in *Xenopus* embryos. However, as the knockdown of DHCR7 clearly shows its requirement in Shh signaling, we considered the possibility that a non-enzymatic activity of DHCR7 is responsible for its inhibition of Shh activity.

Therefore, we treated *DHCR7* mRNA-injected embryos with AY-9944. The embryos developed noticeable microcephaly and a shortened body axis when compared with DHCR7 alone or with AY-

9944 alone (compare Fig. 7C-F; 76%,  $n=58$ ). We also assessed the affects of AY-9944 on Gli reporter activity. Induction of Gli reporter was suppressed more strongly in DHCR7-expressing embryos treated with AY-9944 than that of either AY-9944 or DHCR7 alone (Fig. 7B). These data support the idea that AY9944 augments the antagonistic activity of DHCR7 towards Shh by influencing an activity other than its reductase activity.



**Fig. 4. Loss-of-DHCR7 affects cellular response toward Hh signaling in optic vesicle.** (A-F) DHCR7 knockdown by MOs leads to eye defects. Four-cell stage *Xenopus* embryos were injected marginally in all four blastomeres with 5 ng of control MO (A,D), DHCR7 MOs (B,E) or DHCR7 MOs together with 125 pg of full-length DHCR7 rescue mRNA (C,F). Additional defects detected were in the heart and gut of the swimming tadpole stages (data not shown). (G-R) Loss of DHCR7 affects cellular response towards Hh signaling. Four-cell stage *Xenopus* embryos were injected with 20 ng of control MO (G,G',K,K',O,O'), 20ng of DHCR7 MOs alone (H,H',L,L',P,P'), 50 pg of *Shh-N* mRNA alone (I,I',M,M',Q,Q') or 20 ng of DHCR7 MOs with 50 pg of *Shh-N* mRNA (J,J',N,N',R,R'). Knockdown of DHCR7 reduces *Pax6* expression (G-J), expands the expression of *Pax2* expression (K-N) and upregulates *Gli1* expression (O-R). (G'-R') Continuously treatment of embryos with cyclopamine (100  $\mu$ M) from the blastula stage blocks Hh signaling (G-J,K-N,O-R). Circled areas mark optic vesicles.

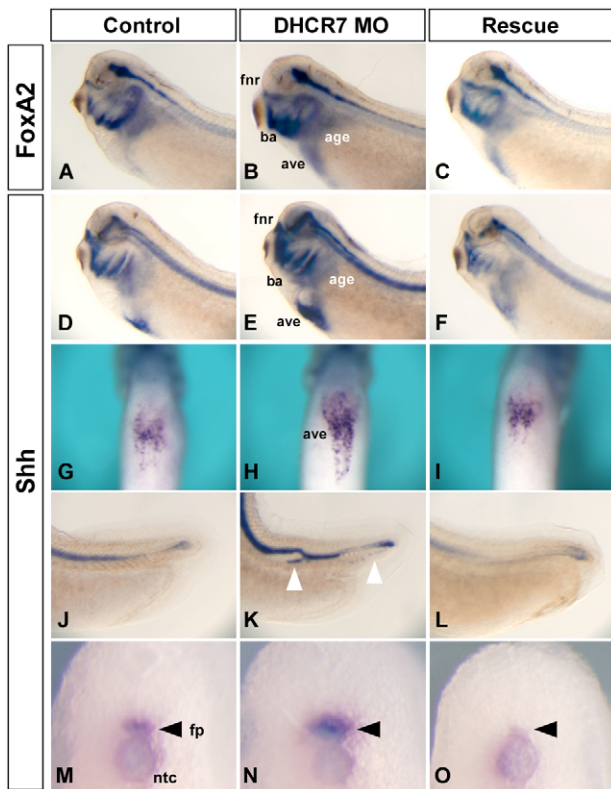
### SLOS-associated mutations reveal dispensability of the sterol-sensing domain for Shh inhibition

In light of our findings both that DHCR7 can act as a Shh inhibitor in *Xenopus* early embryos, and that the reductase activity of DHCR7 is dispensable, we wished to examine the effects of *Xenopus* DHCR7 mutants corresponding to human counterparts found in SLOS (Fig. 7A). One human mutation is DHCR7<sup>R352W</sup> (corresponding to R350W in *Xenopus* DHCR7), which results in abrogation of 95-97% of enzyme activity (Fitzky et al., 2001; Witsch-Baumgartner et al., 2000). Another mutation, DHCR7<sup>IVS8-1G>C</sup>, is the most frequently encountered DHCR7 mutation in individuals with SLOS. This defect causes aberrant splicing of exon 8, generates a truncated protein that is missing part of the sterol sensing domain (SSD; amino acid residues 318-471), and is also significantly reduced in its reductase activity (Fig. 7A). Several missense mutations mapped within the DHCR7 SSD have also been identified among some individuals with SLOS. Overexpression of either DHCR7<sup>R350W</sup> or DHCR7<sup>IVS8-1G>C</sup> resulted in blockade of induction of Gli reporter in the animal cap conjugation assay (Fig. 7L), despite the fact that the mutant proteins have presumably lost essentially all of their reductase activity. Additionally, embryos expressing either DHCR7<sup>IVS8-1G>C</sup> or DHCR7<sup>R350W</sup> displayed severe microcephaly and a shortened body axis (Fig. 7G,H; 90%,  $n=44$  and 78%,  $n=44$ , respectively). When such severely affected embryos were stained for the eye marker, *Pax6*, a single small 'cyclopic' eyespot was detected (Fig. 7K). It is noteworthy that cyclopia is frequently observed in mouse embryos deficient in Shh and in individuals with holoprosencephaly (HPE) (Chiang et al., 1996; Cohen, Jr and Shiota, 2002). The experiments presented in the current study suggest that mutant DHCR7 proteins associated with SLOS are still capable of inhibiting Shh signaling despite their lack of reductase activity.

Next, in order to determine the contribution of the SSD on Hh signaling, we generated a *Xenopus* counterpart (DHCR7<sup>W149X</sup>) of human DHCR7<sup>W151X</sup>, which completely lacks the SSD. Additionally, DHCR7 <sup>$\Delta$ N</sup> lacking most of the N terminus of DHCR7 was generated. When DHCR7<sup>W149X</sup> was expressed, it caused microcephaly in *Xenopus* embryos (Fig. 7I; 70%,  $n=20$ ), and effectively blocked the induction of the Gli-reporter in the animal cap conjugation assay (Fig. 7L), despite the fact that the entire SSD is missing in the mutant protein. Importantly, expression of DHCR7 <sup>$\Delta$ N</sup> did not affect cephalic development (Fig. 7J; 100%,  $n=30$ ) but instead moderately upregulated the expression of the Gli-reporter gene (Fig. 7L). Altogether, these results show that the inhibition of Hh signaling by DHCR7 is mediated via the N-terminal domain of DHCR7.

### Epistasis analysis: DHCR7 acts at the level of Smoothened

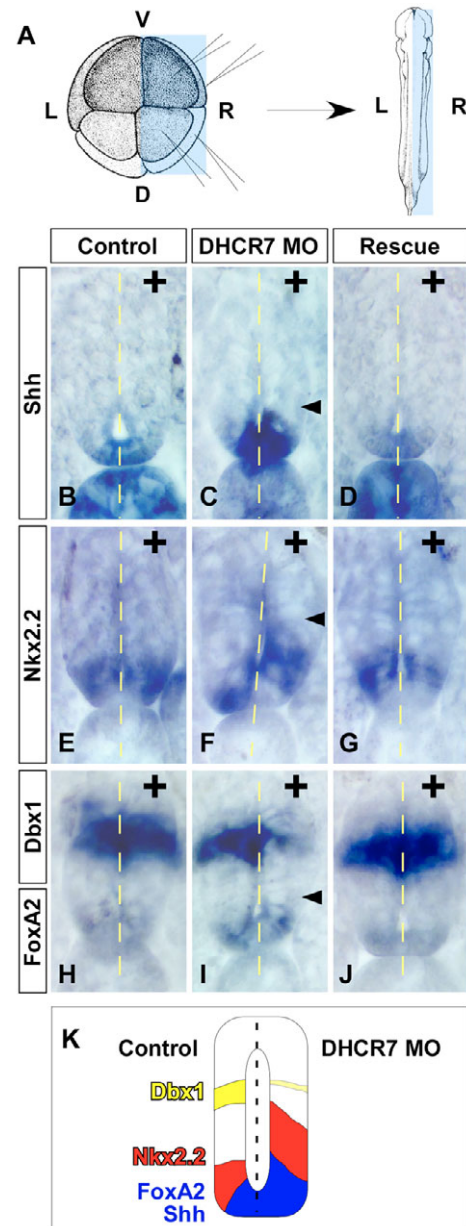
At what level does DHCR7 inhibit Hh signaling? The inhibition by DHCR7 of Hh signaling does not appear to be at the level of cholesterol adduction of the ligand, as loss of reductase activity does not appear to be essential and as Shh-N, which is not cholesterol modified, is also inhibited by DHCR7. Using an animal cap conjugation assay, we also showed that DHCR7 inhibits signaling in Hh responding cells and not at the level of production of the Shh ligand. To determine where in the signal transduction pathway DHCR7 acts, we performed epistasis experiments. First, we examined whether the cyclopic phenotype caused by the expression of DHCR7<sup>R350W</sup> was due to a defect in Shh signaling. The expression patterns of *FoxA2*, *Shh* and *Ptc1* in these embryos were



**Fig. 5. Expansion of *Shh* and *FoxA2* expressing domains in MO-injected embryos.** Four-cell stage *Xenopus* embryos were injected with 20 ng of control MO (A,D,G,J,M), with 20 ng of DHCR7 MOs alone (B,E,H,K,N) or with 500 pg of *DHCR7* rescue mRNA (C,F,I,L,O). Whole-mount in situ hybridization of the tailbud stage embryos microinjected with DHCR7-MOs shows expansion of floor plate as marked by *FoxA2* (A-C) and *Shh* (D-O) expression. (B,E) DHCR7 MOs injected embryos expand *FoxA2* and *Shh* expression domains in frontal nasal region, branchial arches and anterior gut endoderm. (M-O) Transverse sections of embryos indicated in D,E. (K) Ectopic expression of *Shh* was detected in the notochord region of DHCR7 MO injected embryos (40%,  $n=28$ ). ave, anterior ventral endoderm; ba, branchial arches; fl, floor plate (arrowheads); fnr, frontonasal region; age, presumptive anterior gut endoderm. White arrowheads indicate ectopic *Shh* expression.

examined. Injected embryos (Fig. 8) exhibited significant reductions of *FoxA2* (100%,  $n=20$ ), *Shh* (100%,  $n=34$ ) and *Ptc1* (92%,  $n=38$ ) transcripts. We also examined whether dominant-negative Protein Kinase A (dnPKA) could block the midline defects induced by *DHCR7*<sup>R350W</sup>. dnPKA reversed the microcephalic/cyclopic phenotype caused by *DHCR7*<sup>R350W</sup> (Fig. 8G-J; 96%,  $n=46$ ), demonstrating that *DHCR7* acts as an inhibitor of Shh signaling by acting upstream of PKA.

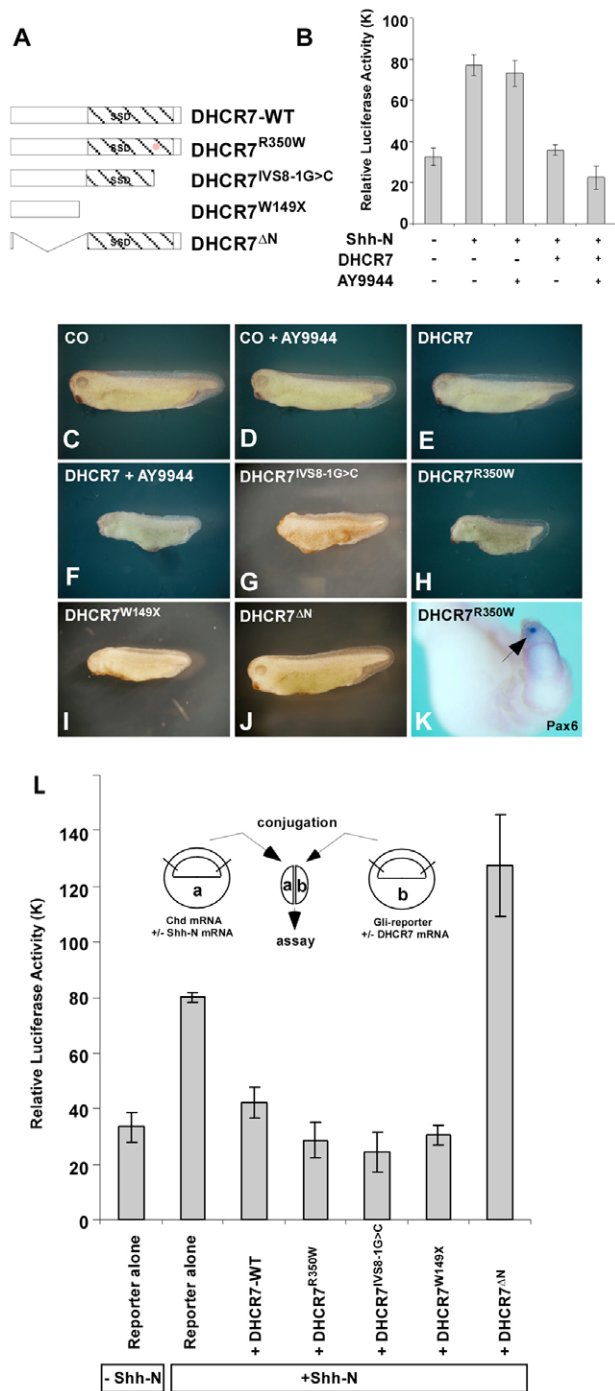
Previous studies have demonstrated that the Hh dependent optic vesicle patterning was inhibited in *Xenopus* embryos treated with the Hh signaling inhibitor cyclopamine, which is known to inhibit the function of Smo (Perron et al., 2003). In order to determine whether *DHCR7* functions upstream or downstream of Smo, embryos injected with *DHCR7* MO were treated with cyclopamine and its effects on eye development were examined. Cyclopamine blocked both the inhibition of *Pax6* and induction of *Pax2* and *Gli1* by *DHCR7*-MO or *Shh-N* overexpression (Fig. 4G'-J',K'-N',O'-R').



**Fig. 6. Neural patterning modulated by DHCR7.** (A) Schema of unilateral injection experiment. Eight-cell stage *Xenopus* embryos were unilaterally (right side) microinjected with 20 ng of control MO (B,E,H) or 20ng of DHCR7 MOs alone (C,F,I) or with 500 pg of *DHCR7* rescue mRNA (D,G,J). Transverse section of whole-mount in situ hybridization of the tailbud stage embryos microinjected with DHCR7-MOs shows ventralization of neural tube as marked by *Shh* (B-D), *Nkx2.2* (E-G), *FoxA2* and *Dbx1* (H-J) expression. Arrowheads indicate the dorsal expression limit of the indicated markers. (K) Summary of ventralization of neural tube by loss of *DHCR7*.

One interpretation of these observations is that *DHCR7* functions to inhibit Hh signaling by acting either at the level of, or upstream of, Smo.

To confirm this possibility, we performed an epistasis experiment using an activated form of Smo (Smo-M2). When embryonic rescue experiments as shown in Fig. 8G-J were performed using Smo-M2, the rescue was only partially successful (data not shown). Similarly, animal cap assays revealed that Smo-M2 partially rescued the



**Fig. 7. Shh signaling and cholesterol synthesis.** (A) Various DHCR7 mutant constructs. (B) Inhibition Hh signaling is enhanced by treatment with AY-9944. Embryos were injected with the reporter construct 8X3'Gli-BS Luc with or without *Shh-N*, *DHCR7* mRNA and treatment with 10  $\mu$ M AY-9944. (C-J) Microinjection of DHCR7 mutants that are defective in reductase activity causes cyclopic phenotypes. The overexpression of *DHCR7* mRNA (E) or treatment with AY-9944 (D) caused minor defects, but a combination of both causes severe defects (F). Embryonic phenotypes caused by microinjection of 1 ng of *DHCR7*<sup>R350W</sup> (H), *DHCR7*<sup>IVS8-1G>C</sup> (G), *DHCR7*<sup>ΔN</sup> (J) and *DHCR7*<sup>W149X</sup> (I) mutants. (K) *Pax6* staining of tailbud stage embryos injected with *DHCR7*<sup>R350W</sup>. Arrow marks 'cyclopic' eye. (L) Modulation of Gli reporter by DHCR7 mutants using animal cap conjugation. Embryos were injected with *Chordin* (with or without *Shh-N* mRNA) or with Gli-BS Luc (with or without *DHCR7* mRNA).

DHCR7-mediated suppression of Hh signaling (Fig. 8K). These observations are consistent with the notion that DHCR7 acts upstream of Smo or at the level of Smo. As *DHCR7*<sup>IVS8-1G>C</sup> is a potent inhibitor of Hh signaling, we next addressed whether *DHCR7*<sup>IVS8-1G>C</sup> could block the activity of Smo-M2. As shown in Fig. 8L, *DHCR7*<sup>IVS8-1G>C</sup> effectively blocked the activation of the Gli reporter gene by Smo-M2, suggesting that DHCR7 affects Hh signaling by impinging on Smo.

## DISCUSSION

We identified *Xenopus* DHCR7 in a microarray screen for genes regulating chordamesodermal development during vertebrate early embryogenesis. Contrary to the prevailing view that DHCR7 functions as a positive regulator of Shh signal transduction, the data presented here instead suggest a novel function for DHCR7 as a negative regulator of Shh signaling. We provide multiple lines of evidence demonstrating that DHCR7 negatively modulates the Shh signaling pathway during early embryogenesis, and suggest that it exerts this action at the level of Smo.

### Requirement for DHCR7 activity during early development

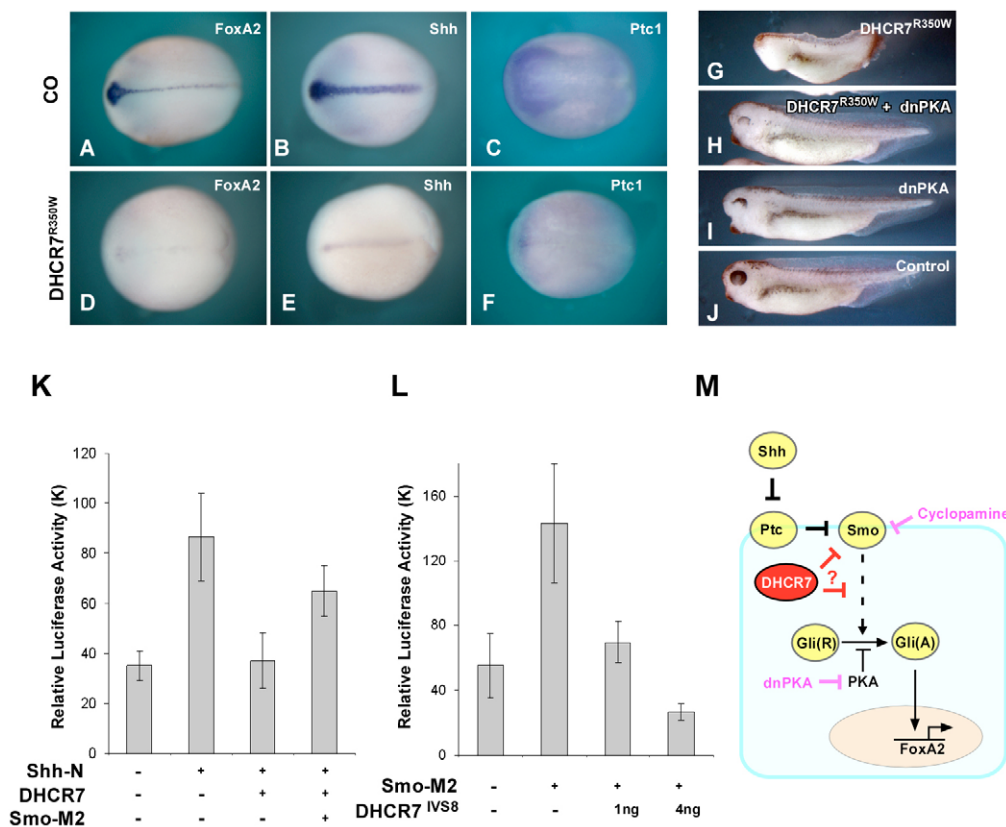
The fact that DHCR7 is a key enzyme in cholesterol biosynthesis, responsible for the final step in the process, would suggest that DHCR7 stimulates Hh signaling through increased production of cholesterol. However, results of our current loss- and gain-of-function analyses do not support this view. Instead, our data indicate that DHCR7 serves to inhibit Hh signaling during early *Xenopus* development. Overexpression of DHCR7 inhibits the effects of ectopic expression of Shh, as well as endogenous Shh activity (Figs 3, 7 and 8), whereas knockdown of DHCR7 by morpholino antisense oligonucleotides leads to enhancement of Shh activity in both the eye and neural tube (Figs 4-6). Importantly, inhibition of the enzymatic activity of DHCR7 either by treatment of embryos with the pharmacological inhibitor AY-9944 or by overexpression of mutant DHCR7 proteins deficient in reductase activity, both still lead to inhibition of Shh signaling (Fig. 7), suggesting that some of the observed effects of DHCR7 on Shh signaling are independent of the enzymatic properties of DHCR7.

Previous work using DHCR7-deficient mouse fibroblasts has suggested that DHCR7 is required for cellular response to Shh (Cooper et al., 2003). This is in contrast to our finding that DHCR7 knockdown promotes Shh signaling (Figs 3, 4), and the reason for this discrepancy is unclear at present. However, it is important to note that the Shh response of fibroblast cells lacking DHCR7 was impaired only when residual endogenous cholesterol was depleted. As embryos from many non-placental vertebrates, including *Xenopus*, stockpile cholesterol maternally [in contrast to mammals, where cholesterol is supplied by yolk sac/placental transfer (Fitzky et al., 2001; Wassif et al., 2001)], differences in cellular cholesterol content may contribute to the difference in DHCR7 loss-of-function phenotypes.

### DHCR7 and Shh signaling in midline development

Shh secreted from the notochord and floor plate forms a concentration gradient along the dorsoventral (DV) axis of the neural tube, thereby controlling the expression of specific homeodomain transcription factors (Ericson et al., 1997). Shh signaling is also negatively regulated by the Hh antagonists Patched1, Rab23, PKA, Hip1 and FKBP8 (Bulgakov et al., 2004; Eggenschwiler et al., 2001; Goodrich et al., 1997; Hammerschmidt et al., 1996; Jeong and McMahon, 2005). Defects in these components lead to





**Fig. 8. The effects of DHCR7 impinges on Smo.** (A-F) Microinjection of *DHCR7*<sup>R350W</sup> mutant inhibits endogenous Shh signaling. Embryos microinjected with 1 ng of *DHCR7*<sup>R350W</sup> mRNA inhibits the expression of *FoxA2*, *Shh* and *Ptc-1*. (G-I) Microinjection of mRNA (500 pg) encoding dnPKA inhibits the cyclopic phenotype caused by *DHCR7*<sup>R350W</sup>. (J) Control uninjected embryo. (K) Smo-M2 rescues DHCR7-mediated Hh signaling inhibition. Embryos were co-injected with 8×3'Gli-BS Luc together with *Chordin*, *Shh-N*, *DHCR7* or *Smo-M2* mRNA (0.5 ng), or in combination, and the reporter gene activity was measured. (L) *DHCR7*<sup>IVS8-1G>C</sup> blocks Gli reporter activation mediated by Smo-M2. Embryos were co-injected with Gli reporter together with *Chordin*, *DHCR7*<sup>IVS8-1G>C</sup> or *Smo-M2* mRNA, or in combination, and reporter gene activity was measured. (M) Model for the role of DHCR7 in Shh signaling. Shh inactivates Ptc, thus permitting the activation of Smo. Activated Smo transmits a Shh signal to activate Gli protein. PKA inhibits Gli activation. DHCR7 inhibits Shh signaling either at the level of or downstream of Smo.

ventralization of the neural tube. In the present study, a similarly ventralized neural tube phenotype was also observed in *Xenopus* embryos when DHCR7 activity was inhibited (Figs 5 and 6). We note that mice deficient in DHCR7 do not display obvious defects in the developing central nervous system (Fitzky et al., 2001; Wassif et al., 2001), but our findings are consistent with the recent observation that the formation of serotonin (5-HT) neurons, which is dependent on high Shh signaling, showed a threefold increase in the number of neurons in the ventral CNS of *DHCR7*<sup>Δ3-5</sup> mutant mice (Waage-Baudet et al., 2003). In addition to its role in the CNS, Shh is also required in the formation of the notochord and for maintenance of Shh signaling in that tissue once formed (Chiang et al., 1996). That DHCR7 is expressed both in the notochord and ventral CNS, and that the loss of DHCR7 enhances the transcription of *Shh* and *FoxA2* in these tissues (Fig. 5), lead us to surmise that DHCR7 functions to negatively regulate levels of Shh signaling in both these structures.

### What is the molecular mechanism underlying Shh inhibition by DHCR7?

We propose that DHCR7 inhibits Hh signaling at the level or downstream of Smo based on the following observations. First, the primary effects of DHCR7 do not appear to occur at the level of regulating the transcription of the Shh because DHCR7 was able to inhibit the effects of overexpression of Shh. Second, the inhibition

of Hh signaling by DHCR7 appears to be independent of cholesterol adduction of the ligand, as Shh-N, which is not cholesterol modified, is also inhibited by DHCR7. Third, an animal cap conjugation assay demonstrated that DHCR7 inhibits signaling in Hh-responding cells and not at the level of production of the ligand. Fourth, epistasis analysis using Smo-M2 and *DHCR7*<sup>IVS8-1G>C</sup> is consistent with the notion that DHCR7 regulates Shh signaling either at the level of Smo or impinges downstream of the Smo. We also observed that dnPKA reversed the microcephalic/cyclopic phenotype caused by *DHCR7*<sup>R350W</sup>, demonstrating that DHCR7 acts as an inhibitor of Hh signaling by acting upstream of PKA.

DHCR7 functions intracellularly at the level or downstream of Smo and possesses a conserved sterol-sensing domain (SSD) that is found predominantly among proteins involved in vesicular trafficking (Kuwabara and Labouesse, 2002). Ptc1 and Disp1 are implicated in the vesicular trafficking of Hh, whereas SCAP, NPC1 and NPC1L are involved in sterol trafficking. Accordingly, fibroblasts isolated from many individuals with SLOS show abnormal storage of sterols, presumably resulting from defects in sterol trafficking (Wassif et al., 2002). Given that the Ptc protein is largely detected within intracellular vesicles (Incardona et al., 2000), and that both Ptc and Smo appear to shuttle between the plasma membrane and cytoplasmic vesicles in response to the presence/absence of Hh ligand (Denef et al., 2000), it is tempting to speculate

that DHCR7 participates in the regulation of vesicular trafficking of Hh signaling components. However, our current DHCR7 mutational analyses confound this simple interpretation, as a DHCR7 mutant containing an intact N terminus (but lacking the SSD) is sufficient to inhibit the action of Hh signaling. It is currently unclear what the role of this N-terminal region might be, and further work will be required to fully understand the function of each domain of DHCR7.

### The role of DHCR7 in the pathogenesis of SLOS

One curious aspect of SLOS is the wide range of symptoms seen in this disease. Individuals appear to display opposing phenotypes for the same underlying genetic condition. For example, some individuals are polydactyl (extra digits), while others are monodactyl (a single digit); some show holoprosencephaly, while others are macrocephalic (Kelley and Hennekam, 2000). How can mutations in a single gene cause such seemingly divergent phenotypes? As DHCR7 appears to have two distinct activities, a Hh antagonistic activity and a reductase enzymatic activity, perhaps, depending on the precise nature of the specific mutations, the activity of DHCR7 is oppositely modulated to bring phenotypes of opposing directionality. Additionally, mutations in *DHCR7* can have the effect of altering intracellular sterol concentrations, which may affect cellular responses to Hh signaling (Cooper et al., 2003).

An important topic to address in the future is the biochemical nature of the *DHCR7* mutants, especially how point mutations in the SSD domain or deletions affect the antagonistic activity of Shh relative to its reductase activity. Additionally, it is important to address how DHCR7 expression is regulated and its relationship to the occurrence of SLOS as proper expression of DHCR7 is essential for normal midline development.

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### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/133/12/2395/DC1>

### References

- Blitz, I. L. and Cho, K. W. (1995). Anterior neurectoderm is progressively induced during gastrulation: the role of the *Xenopus* homeobox gene orthodenticle. *Development* **121**, 993-1004.
- Blitz, I. L., Shimmi, O., Wunnenberg-Stapleton, K., O'Connor, M. B. and Cho, K. W. (2000). Is chordin a long-range- or short-range-acting factor? Roles for BMP1-related metalloproteases in chordin and BMP4 autoregulatory loop regulation. *Dev. Biol.* **223**, 120-138.
- Briscoe, J., Pierani, A., Jessell, T. M. and Ericson, J. (2000). A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell* **101**, 435-445.
- Bulgakov, O. V., Eggenschwiler, J. T., Hong, D. H., Anderson, K. V. and Li, T. (2004). FKBP8 is a negative regulator of mouse sonic hedgehog signaling in neural tissues. *Development* **131**, 2149-2159.
- Chamoun, Z., Mann, R. K., Nellen, D., von Kessler, D. P., Bellotto, M., Beachy, P. A. and Basler, K. (2001). Skinny hedgehog, an acyltransferase required for palmitoylation and activity of the hedgehog signal. *Science* **293**, 2080-2084.
- Chen, J. K., Taipale, J., Cooper, M. K. and Beachy, P. A. (2002). Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes Dev.* **16**, 2743-2748.
- Chen, M. H., Li, Y. J., Kawakami, T., Xu, S. M. and Chuang, P. T. (2004). Palmitoylation is required for the production of a soluble multimeric Hedgehog protein complex and long-range signaling in vertebrates. *Genes Dev.* **18**, 641-659.
- Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407-413.
- Cho, K. W. Y., Blumberg, B., Steinbeisser, H. and De Robertis, E. (1991). Molecular nature of Spemann's organizer: the role of the *Xenopus* homeobox gene goosecoid. *Cell* **67**, 1111-1120.
- Cohen, M. M., Jr and Shiota, K. (2002). Teratogenesis of holoprosencephaly. *Am. J. Med. Genet.* **109**, 1-15.
- Cooper, M. K., Porter, J. A., Young, K. E. and Beachy, P. A. (1998). Teratogen-mediated inhibition of target tissue response to Shh signaling. *Science* **280**, 1603-1607.
- Cooper, M. K., Wassif, C. A., Krakowiak, P. A., Taipale, J., Gong, R., Kelley, R. L., Porter, F. D. and Beachy, P. A. (2003). A defective response to Hedgehog signaling in disorders of cholesterol biosynthesis. *Nat. Genet.* **33**, 508-513.
- Dakubo, G. D., Wang, Y. P., Mazerolle, C., Campsall, K., McMahon, A. P. and Wallace, V. A. (2003). Retinal ganglion cell-derived sonic hedgehog signaling is required for optic disc and stalk neuroepithelial cell development. *Development* **130**, 2967-2980.
- Denef, N., Neubuser, D., Perez, L. and Cohen, S. M. (2000). Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened. *Cell* **102**, 521-531.
- Eggenschwiler, J. T., Espinoza, E. and Anderson, K. V. (2001). Rab23 is an essential negative regulator of the mouse Sonic hedgehog signalling pathway. *Nature* **412**, 194-198.
- Ekker, S. C., McGrew, L. L., Lai, C. J., Lee, J. J., von Kessler, D. P., Moon, R. T. and Beachy, P. A. (1995a). Distinct expression and shared activities of members of the hedgehog gene family of *Xenopus laevis*. *Development* **121**, 2337-2347.
- Ekker, S. C., Ungar, A. R., Greenstein, P., von Kessler, D. P., Porter, J. A., Moon, R. T. and Beachy, P. A. (1995b). Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. *Curr. Biol.* **5**, 944-955.
- Ericson, J., Briscoe, J., Rashbass, P., van Heyningen, V. and Jessell, T. M. (1997). Graded sonic hedgehog signaling and the specification of cell fate in the ventral neural tube. *Cold Spring Harb. Symp. Quant. Biol.* **62**, 451-466.
- Fitzky, B. U., Witsch-Baumgartner, M., Erdel, M., Lee, J. N., Paik, Y. K., Glossmann, H., Utermann, G. and Moebius, F. F. (1998). Mutations in the Delta7-sterol reductase gene in patients with the Smith-Lemli-Opitz syndrome. *Proc. Natl. Acad. Sci. USA* **95**, 8181-8186.
- Fitzky, B. U., Moebius, F. F., Asaoka, H., Waage-Baudet, H., Xu, L., Xu, G., Maeda, N., Kluckman, K., Hiller, S., Yu, H. et al. (2001). 7-Dehydrocholesterol-dependent proteolysis of HMG-CoA reductase suppresses sterol biosynthesis in a mouse model of Smith-Lemli-Opitz/RSH syndrome. *J. Clin. Invest.* **108**, 905-915.
- Goodrich, L. V., Milenkovic, L., Higgins, K. M. and Scott, M. P. (1997). Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science* **277**, 1109-1113.
- Guy, R. K. (2000). Inhibition of sonic hedgehog autoprocessing in cultured mammalian cells by sterol deprivation. *Proc. Natl. Acad. Sci. USA* **97**, 7307-7312.
- Hammerschmidt, M., Bitgood, M. J. and McMahon, A. P. (1996). Protein kinase A is a common negative regulator of Hedgehog signaling in the vertebrate embryo. *Genes Dev.* **10**, 647-658.
- Harland, R. M. (1991). In situ hybridization: an improved whole-mount method for *Xenopus* embryos. *Methods Cell Biol.* **36**, 685-695.
- Incardona, J. P., Gaffield, W., Kapur, R. P. and Roelink, H. (1998). The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction. *Development* **125**, 3553-3562.
- Incardona, J. P., Lee, J. H., Robertson, C. P., Enga, K., Kapur, R. P. and Roelink, H. (2000). Receptor-mediated endocytosis of soluble and membrane-tethered Sonic hedgehog by Patched-1. *Proc. Natl. Acad. Sci. USA* **97**, 12044-12049.
- Ingham, P. W. and McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* **15**, 3059-3087.
- Jeong, J. and McMahon, A. P. (2005). Growth and pattern of the mammalian neural tube are governed by partially overlapping feedback activities of the hedgehog antagonists patched 1 and Hhip1. *Development* **132**, 143-154.
- Kelley, R. L. and Hennekam, R. C. (2000). The Smith-Lemli-Opitz syndrome. *J. Med. Genet.* **37**, 321-335.
- Kelley, R. L., Roessler, E., Hennekam, R. C., Feldman, G. L., Kosaki, K., Jones, M. C., Palumbos, J. C. and Muenke, M. (1996). Holoprosencephaly in RSH/Smith-Lemli-Opitz syndrome: does abnormal cholesterol metabolism affect the function of Sonic Hedgehog? *Am. J. Med. Genet.* **66**, 478-484.
- Koebnick, K., Hollemann, T. and Pieler, T. (2003). A restrictive role for Hedgehog signalling during otic specification in *Xenopus*. *Dev. Biol.* **260**, 325-338.
- Kuwabara, P. E. and Labouesse, M. (2002). The sterol-sensing domain: multiple families, a unique role? *Trends Genet.* **18**, 193-201.
- Lai, C. J., Ekker, S. C., Beachy, P. A. and Moon, R. T. (1995). Patterning of the neural ectoderm of *Xenopus laevis* by the amino-terminal product of hedgehog autoproteolytic cleavage. *Development* **121**, 2349-2360.
- Lee, J. J., Ekker, S. C., von Kessler, D. P., Porter, J. A., Sun, B. I. and Beachy, P. A. (1994). Autoproteolysis in hedgehog protein biogenesis. *Science* **266**, 1528-1537.
- Lewis, P. M., Dunn, M. P., McMahon, J. A., Logan, M., Martin, J. F., St-Jacques, B. and McMahon, A. P. (2001). Cholesterol modification of sonic

- hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1. *Cell* **105**, 599-612.
- Lum, L. and Beachy, P. A.** (2004). The Hedgehog response network: sensors, switches, and routers. *Science* **304**, 1755-1759.
- Lupo, G., Liu, Y., Qiu, R., Chandraratna, R. A., Barsacchi, G., He, R. Q. and Harris, W. A.** (2005). Dorsoventral patterning of the *Xenopus* eye: a collaboration of Retinoid, Hedgehog and FGF receptor signaling. *Development* **132**, 1737-1748.
- Macdonald, R., Barth, K. A., Xu, Q., Holder, N., Mikkola, I. and Wilson, S. W.** (1995). Midline signalling is required for Pax gene regulation and patterning of the eyes. *Development* **121**, 3267-3278.
- Pepinsky, R. B., Zeng, C., Wen, D., Rayhorn, P., Baker, D. P., Williams, K. P., Bixler, S. A., Ambrose, C. M., Garber, E. A., Miatkowski, K. et al.** (1998). Identification of a palmitic acid-modified form of human Sonic hedgehog. *J. Biol. Chem.* **273**, 14037-14045.
- Perron, M., Boy, S., Amato, M. A., Viczian, A., Koebernick, K., Pieler, T. and Harris, W. A.** (2003). A novel function for Hedgehog signalling in retinal pigment epithelium differentiation. *Development* **130**, 1565-1577.
- Pierani, A., Brenner-Morton, S., Chiang, C. and Jessell, T. M.** (1999). A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* **97**, 903-915.
- Porter, J. A., Ekker, S. C., Park, W. J., von Kessler, D. P., Young, K. E., Chen, C. H., Ma, Y., Woods, A. S., Cotter, R. J., Koonin, E. V. et al.** (1996). Hedgehog patterning activity: role of a lipophilic modification mediated by the carboxy-terminal autoprocessing domain. *Cell* **86**, 21-34.
- Roux, C. and Aubry, M.** (1966). [Teratogenic action in the rat of an inhibitor of cholesterol synthesis, AY 9944]. *C. R. Seances Soc. Biol. Fil.* **160**, 1353-1357.
- Sasai, Y., Lu, B., Steinbeisser, H. and De Robertis, E. M.** (1995). Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in *Xenopus*. *Nature* **376**, 333-336.
- Sasaki, H., Hui, C., Nakafuku, M. and Kondoh, H.** (1997). A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. *Development* **124**, 1313-1322.
- Shin, Y., Kitayama, A., Koide, T., Peiffer, D. A., Mochii, M., Liao, A., Ueno, N. and Cho, K. W.** (2005). Identification of neural genes using *Xenopus* DNA microarrays. *Dev. Dyn.* **232**, 432-444.
- Tsuda, H., Sasai, N., Matsuo-Takasaki, M., Sakuragi, M., Murakami, Y. and Sasai, Y.** (2002). Dorsalization of the neural tube by *Xenopus* tiarin, a novel patterning factor secreted by the flanking nonneural head ectoderm. *Neuron* **33**, 515-528.
- Tsuji, S. and Hashimoto, C.** (2005). Choice of either beta-catenin or Groucho/TLE as a co-factor for Xtcf-3 determines dorsal-ventral cell fate of diencephalon during *Xenopus* development. *Dev. Genes Evol.* **215**, 275-284.
- Turner, D. L. and Weintraub, H.** (1994). Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.* **8**, 1434-1447.
- Ungar, A. R. and Moon, R. T.** (1996). Inhibition of protein kinase A phenocopies ectopic expression of hedgehog in the CNS of wild-type and cyclops mutant embryos. *Dev. Biol.* **178**, 186-191.
- Waage-Baudet, H., Lauder, J. M., Dehart, D. B., Kluckman, K., Hiller, S., Tint, G. S. and Sulik, K. K.** (2003). Abnormal serotonergic development in a mouse model for the Smith-Lemli-Opitz syndrome: implications for autism. *Int. J. Dev. Neurosci.* **21**, 451-459.
- Wang, E., Miller, L. D., Ohnmacht, G. A., Liu, E. T. and Marincola, F. M.** (2000). High-fidelity mRNA amplification for gene profiling. *Nat. Biotechnol.* **18**, 457-459.
- Wassif, C. A., Maslen, C., Kachilele-Linjewile, S., Lin, D., Linck, L. M., Connor, W. E., Steiner, R. D. and Porter, F. D.** (1998). Mutations in the human sterol delta7-reductase gene at 11q12-13 cause Smith-Lemli-Opitz syndrome. *Am. J. Hum. Genet.* **63**, 55-62.
- Wassif, C. A., Zhu, P., Kratz, L., Krakowiak, P. A., Battaile, K. P., Weight, F. F., Grinberg, A., Steiner, R. D., Nwokoro, N. A., Kelley, R. I. et al.** (2001). Biochemical, phenotypic and neurophysiological characterization of a genetic mouse model of RSH/Smith-Lemli-Opitz syndrome. *Hum. Mol. Genet.* **10**, 555-564.
- Wassif, C. A., Vied, D., Tsokos, M., Connor, W. E., Steiner, R. D. and Porter, F. D.** (2002). Cholesterol storage defect in RSH/Smith-Lemli-Opitz syndrome fibroblasts. *Mol. Genet. Metab.* **75**, 325-334.
- Witsch-Baumgartner, M., Fitzky, B. U., Ogorelkova, M., Kraft, H. G., Moebius, F. F., Glossmann, H., Seedorf, U., Gillissen-Kaesbach, G., Hoffmann, G. F., Clayton, P. et al.** (2000). Mutational spectrum in the Delta7-sterol reductase gene and genotype-phenotype correlation in 84 patients with Smith-Lemli-Opitz syndrome. *Am. J. Hum. Genet.* **66**, 402-412.
- Zeng, X., Goetz, J. A., Suber, L. M., Scott, W. J., Jr, Schreiner, C. M. and Robbins, D. J.** (2001). A freely diffusible form of Sonic hedgehog mediates long-range signalling. *Nature* **411**, 716-720.