Cholesterol Metabolism Is Required for Intracellular Hedgehog Signal Transduction *In Vivo*

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Abstract

We describe the *rudolph* mouse, a mutant with striking defects in both central nervous system and skeletal development. *Rudolph* is an allele of the cholesterol biosynthetic enzyme, hydroxysteroid (17-beta) dehydrogenase 7, which is an intriguing finding given the recent implication of oxysterols in mediating intracellular Hedgehog (*Hh*) signaling. We see an abnormal sterol profile and decreased *Hh* target gene induction in the *rudolph* mutant, both *in vivo* and *in vitro*. Reduced *Hh* signaling has been proposed to contribute to the phenotypes of congenital diseases of cholesterol metabolism. Recent *in vitro* and pharmacological data also indicate a requirement for intracellular cholesterol synthesis for proper regulation of *Hh* activity via Smoothened. The data presented here are the first *in vivo* genetic evidence supporting both of these hypotheses, revealing a role for embryonic cholesterol metabolism in both CNS development and normal *Hh* signaling.

Introduction

**Hedgehog** (*Hh*) ligands have numerous and fundamental roles in both embryonic development [1,2] and tumor biology [3]. Mammalian hedgehog proteins bind to the transmembrane receptor *Patched* (*Ptch*) and thereby relieve its repression of *Smoothened* (*Smo*). Intracellular transduction of *Smo* activity requires processing of GLI proteins. The primary cilium has shown to be an essential structural component for proper *Hh* signaling in mammals with dynamic localization of *Ptch* in response to binding of Sonic hedgehog (*Shh*; [4,5,6,7]). Functional *Hh* signaling requires removal of *Ptch* from the cilium, translocation of *Smo* to the cilium, and activation of *Smo* by an as yet unknown mechanism [5,6,8,9].

It has been well established that cholesterol is an essential component of *Hh* signal transduction. Processing of the *Hh* ligand in the producing cell includes the covalent modification of cholesterol to the carboxyl end of the immature protein. Although cholesterol in *Hh* proteins is thought to facilitate the proper dispersal of SHH through the target field, it is not necessary for signal transduction [7]. More recently, however, metabolites of cholesterol and cholesterol biosynthetic pathway intermediates have been shown to have an intracellular role in *Hh* signal transduction. For example, pharmacological inhibition of cholesterol biosynthesis leads to defective responses to *Hh* ligand, independent of SHH processing, both *in vitro* and *in vivo*, with the *in vivo* effect mimicking *Shh* loss of function phenotypes [10,11,12]. Moreover, not only can cholesterol-derived oxysterols activate the *Hh* pathway *in vitro* [13], but treatment of cells with oxysterols has been shown to cause translocation of *Ptch* and *Smo* to the cilium in the absence of SHH ligand [8].

Mutations in enzymes required for cholesterol biosynthesis are associated with a number of human diseases [14]. The best known is Smith-Lemli-Opitz Syndrome, in which patients have central nervous system (CNS) malformations, including holoprosencephaly and microcephaly, and skeletal defects (most often postaxial polydactyly) caused by mutations in *7-dehydrocholesterol reductase* (*DHCR7*) [15]. Other disorders of cholesterol biosynthesis include desmosterolosis, lathosterolosis, X-linked dominant chondrodysplasia punctata (*CDPX2*), CHILD syndrome (congenital hemidysplasia with ichthyosiform erythroderma or nevus and limb defects) and Greenberg skeletal dysplasia. Overlapping features of these disorders include abnormalities in the CNS, facial dysmorphisms, and skeletal defects, often including polydactyly or other digit patterning defects. Mouse models also exist for many of these disorders and have similar defects. The frequent occurrence in these human syndromes and mouse mutants of defects in neurodevelopment, craniofacial morphogenesis and skeletal growth and patterning has led to the proposal that abnormal *Hh* signaling may be the root cause of these embryological defects. This speculation is based principally on genetic experiments that have shown a role for *Hh* signaling in the development of all of these tissues, and the known role of sterol metabolism in *Hh* signal transduction. Despite this, there is relatively little direct evidence from these mouse models for a defect in *Hh* signaling.

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Author Summary

The molecules and signaling pathways that regulate growth and patterning of the developing embryo are still being elucidated, and one valuable experimental approach is the use of animal models, such as the mouse. We have identified a recessive mutation in the mouse, rudolph, that causes abnormal forebrain development and have determined that the mutated gene encodes hydroxysteroid (17-beta) dehydrogenase 7, an enzyme necessary for cholesterol biosynthesis. Cholesterol is essential for proper signal transduction of the hedgehog family of proteins, key regulators of both developmental biology and tumor progression. We show that hedgehog signaling is diminished in our rudolph mutant. Our conclusions from studying this mouse mutant support two recent hypotheses in developmental biology. First, several human malformation syndromes are known to be caused by defects in cholesterol metabolism, but support linking the malfunction to abnormal hedgehog signaling has not definitively been made. Second, while in vitro studies have shown that proper levels of metabolic by-products of cholesterol are necessary for proper hedgehog signaling, our studies offer the strongest genetic animal model evidence to support this idea.

Here we describe the phenotype of the rudolph mouse mutant, an ethyl-nitrosourea (ENU)-induced mutation in hydroxysteroid (17-beta) dehydrogenase 7 (Hsd17b7), which was the last enzyme of the cholesterol biosynthetic pathway to be identified and one of four proteins of the sterol-4-demethylase complex [16]. Rudolph mutants have severe developmental abnormalities in several tissues including the brain and appendicular skeleton. We find that tissues from rudolph mutants have an abnormal sterol profile consistent with impaired activity of the sterol-4-demethylase complex. We further demonstrate that the rudolph mutant has deficient responses to Hh signaling, both in vivo and in vitro. These results support the recently proposed model that functional intracellular sterol metabolism is required for proper cilia-mediated activation of the Hh signaling pathway.

Results

Rudolph mutants show defective growth and patterning of the CNS and appendicular skeleton

We recently recovered the rudolph mutation via an ENU mutagenesis screen designed to identify recessive mutations affecting development of the mammalian forebrain. Rudolph mutants were first ascertained by a blood spot on the end of the nose and their abnormally curved forelimbs (Figure 1B). The precursor to this nasal phenotype was sometimes evident at earlier stages as a blebbing of the craniofacial epithelium (Figure S1A). Examination of the embryonic skeleton revealed that all long bones of the appendicular skeleton were significantly shorter than those of wild-type littersmates while the axial skeleton and ribs appeared normal (Figure 1D, Figure S2, Table S1). Further analysis of the embryos revealed severe defects in CNS development. The telencephalic tissue was markedly reduced in size and highly disorganized in mutants at embryonic day (E) 16.5 (Figure 1F, 1H). Mutants had a smaller neurogenic ventricular zone and clumps of cells in the developing cortical plate. Similar defects were seen in the E16.5 retina and spinal cord (Figure 1J, Figure S3B). Initial cortical morphogenesis appeared largely normal (Figure S3D, S3F).

To further characterize the rudolph phenotype, we performed a molecular analysis of the cortical phenotype. We assessed cell proliferation at E14.5 by BrdU treatment of pregnant dams or immunostaining of embryos with Ki67 and found a marked decrease in proliferation in mutants (Figure 2B). Some of the mitoses we detected were seen as foci of BrdU-positive cells (inset in Figure 2B). We thus interpreted the clusters of cells seen histologically in the rud cortex to be neurogenic foci. To determine the cause of the reduced neuronal tissue, we assayed apoptosis at E14.5 using the TUNEL reaction and found increased levels of cell death in the mutant tissue, distributed throughout the cortex and enriched along the ventricular surface (Figure 2D). An increased level of cell death was not seen in non-neural tissue (data not shown). Decreased, disorganized neuronal proliferation and increased cell death were also evident at E12.5 (data not shown). Immunohistochemistry for Tuj1 to identify differentiated neurons at E14.5 showed a marked decrease in differentiation in mutants compared to wild-type (Figure 2F). In addition, foci of Tuj1 immunoreactivity appeared in regions of no differentiation, consistent with the disorganization of the cortex seen histologically. Disorganized proliferation was also evident in the developing rudolph retina, where we observed similarly abnormal neuronal differentiation, decreased cell proliferation and increased apoptosis, but at different stages of development. Whereas at E12.5 we saw no significant decrease in proliferation or apoptosis between wild-type and mutant (Figure 2H; data not shown), at E14.5 we found a decrease in BrdU incorporation in the mutant retina (Figure 2J) and an increase in apoptosis (Figure 2L). Furthermore, the pattern of neuronal disorganization we saw in the rud cortex was similarly evident in the rud retina at E14.5 (Figure 2N).

Genetic background affects the rudolph phenotype

The phenotypes of the rudolph mutants exhibit a variability in severity that appears to be dependent on genetic background. For example, we noted increased blebbing on the developing head and limbs in embryos that came from a mixed background (Figure S1B–S1D). Embryos from our ENU screen have a mixed genetic background with contributions coming from both A/J (the mutagenized strain) and FVB mice (introduced as part of the outcross for mapping purposes). In this A/J; FVB background, embryos were recovered in approximately Mendelian ratios from E10.5–E18.5 (Table S2). No mutants have been recovered after birth, suggesting they are among the stillborn fetuses. Upon introducing the B6 background, the number of mutant embryos did not decrease significantly, but we then began to see a number of dead embryos from E11.5 and older (7.8%), an increase in the severity of the nasal blebbing (Figure S1A–S1C: 14.3%) and limb patterning defects (10.4%). Further introgression into the B6 background resulted in no significant decrease in recovery of mutant embryos but a large increase in the incidence of more severe blebbing (48.5% in pooled N1, N2, and N3B6 mice). Backcrossing to FVB rescued this defect, and blebbing is essentially absent in N3 FVB mice. The reduced fecundity of 129 mice limited our analysis of this genetic background, preventing any definitive comments on modifiers on the 129 background (Table S2).

Hsd17b7 is the gene mutated in rudolph embryos

We initially mapped the rudolph mutation to Chromosome 1 using a whole-genome 768-marker single nucleotide polymorphism (SNP) panel [17]. Examination of the 255 predicted and known genes in the region suggested Hsd17b7 as a candidate for further analysis because of its known function in cholesterol metabolism and its reported expression pattern in limb buds and
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the developing nervous system [16]. Sequencing of Hsd17b7 revealed a point mutation in the sixth intron, 27 base pairs upstream of the intron-exon boundary (Figure 3A). We analyzed transcripts by RT-PCR with primers spanning exon 7 and found the predominant PCR product in mutant tissue to be smaller than that of wild type (Figure 3B). Sequencing of this product revealed a precise excision of the seventh exon in the smaller PCR species. The loss of exon 7 was confirmed with primers in the sixth and seventh exons (Figure 3C). Interestingly, a small amount of this truncated transcript was present in wild-type cDNA, and, conversely, mutant tissues retained a very small fraction of the wild-type transcript (Figure 3B, 3C). We hypothesize that these RT-PCR products represent two naturally occurring forms of the Hsd17b7 transcript and that the rudolph ENU mutation affects the ratio of their abundances. The phenotypes we observe in the rudolph mutants appear to be somewhat tissue specific and have differing expressivity in different strains. However, the variation in the cDNA splicing pattern does not differ among different tissues examined (heart/lung, limbs, brain, or whole embryo) or depend on varying genetic backgrounds, suggesting that tissue specific transcription and variation in genetic background account for the variability in phenotype (Figure S1E).

Loss of the seventh exon of HSD17B7 is predicted to encode a protein with an in-frame deletion of 19 amino acids. To assess the effect of this splicing mutation, we tested HSD17B7 expression by Western immunoblot analysis and found only trace levels of the protein in mutant tissue lysates (Figure 3D). Although the deleted seventh exon is part of a putative endoplasmic reticulum anchoring sequence, in vitro expression of a rud-GFP fusion protein results in deficient protein rather than mislocalization, suggesting that the rudolph deletion generates an unstable protein product (Figure S4). Embryos homozygous for a null allele of Hsd17b7 generated from a 129 genetic background do not survive past E10.5, suggesting the rudolph allele is likely a hypomorph [18,19].

**Figure 1. Phenotypic characterization of rudolph mutants.** Rudolph mutants (B) are readily distinguishable from wild-type (A) littermates at E16.5 by their shortened, curved forelimbs. Some mutants have accumulations of blood in nasal blebs (B). Skeletal preparations of E16.5 limbs show significantly shorter long bones in mutants (D). The mutant forebrain at E16.5 (F) is severely affected with a reduction in tissue throughout. Cleft palate is also noted in mutants (arrow in F). While the wild-type cortex (G) has a readily identifiable ventricular zone (vz), intermediate zone (iz), and cortical plate (cp), closer examination of the dorsal cortex in the mutant reveals significant disorganization (H). Similar disorganization is seen in the rud retina (J).

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**Figure 2. Telencephalic and retinal development are abnormal in rudolph mutants.** (A,B) BrdU labeling and immunohistochemistry shows a significant decrease in dividing cells in the rudolph cortex (A) as compared to the wild-type (A). The inset in B highlights the groups of dividing cells sometimes seen away from the ventricular zone. (C,D) Analysis of cell death using the TUNEL assay reveals a large increase in cell death in mutant tissue (B) with an enrichment of the death at the ventricular surface. (E,F) Neuronal differentiation as measured by TuJ1 immunohistochemistry is dramatically decreased in rud mutants (F). (G,H) BrdU labeling at E12.5 does not indicate a significant difference in rates of cell division in the rudolph retina at E12.5, but a significant decrease is seen at E14.5 (J). Similar to cortical development, the decreased proliferation is accompanied by an increase in apoptosis as shown by the TUNEL assay (K,L). Neuronal differentiation in the mutant retina is disorganized in mutant tissue (M) as compared to wild-type (N).

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Figure 3. *Hsd17b7* is the gene carrying the *rudolph* mutation. (A) Sequencing of the *Hsd17b7* gene revealed a thymidine to adenosine mutation in the intron of *rudolph* mutants between the sixth and seventh exons. (B,C) RT-PCR analysis of wild-type and mutant transcripts with primers for exons 6–8 of the *rudolph* transcript give two PCR products. The larger species is found in low quantities in the *rud* mutant tissue. A smaller species, which lacks the seventh exon, was also amplified. RT-PCR with primers for the sixth and seventh exon results in very little amplification of mutant cDNA. (D) Immunoblotting from embryo lysates shows very little protein in *rudolph* mutants as compared to wild-type.

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Sterol profiles are abnormal in *rudolph* tissues

We analyzed the sterols present in liver and brain tissue from wild-type, heterozygous, and *rudolph* embryos at E12.5 by gas chromatography-mass spectrometry (Figure 4) and found marked differences between wild-type and mutant tissues. The identified abnormalities in methylsterol abundances are consistent with reduced function of the Hsd1b7 enzyme in *rudolph* brain tissues (Table 1). Sterol species upstream of Hsd17b7 activity were present in increased amounts, the most prominent of which were the HSD17B7 substrates zymosterone and 4α-methyl-zymosterone, and a third ketosterol tentatively identified as methylcholest-7-en-3-one. Various mono- and dimethylsterols that do not normally accumulate in wild-type tissues were also present in increased amounts, including 4α-methyl-5α-cholesta-8(9),24-dien-3β-ol (j), 4,4α-dimethyl-5α-cholesta-8(9),24-dien-3β-ol (i), and methylcholest-7-en-3-one (tentative) (k).

Hedgehog signaling is disrupted in the *rudolph* mutant central nervous system

In view of the important role of Hh signaling in patterning of the limbs, face, and brain, and the genetic and cellular evidence for a role of cholesterol metabolism in this pathway, we hypothesized that Hh signaling is perturbed in the *rudolph* mutant. Furthermore, recent evidence specifically implicates intracellular sterols in the regulation of the subcellular localization of the Hh signaling components, Patched and Smoothened, supporting the possibility that an abnormal sterol profile in *rudolph* mutants could disrupt Hh signaling [5,6,8]. To assess this, we generated mice homozygous for the *rudolph* mutation that carried the Patched-lacZ gene, a transcriptional target of Gli2 and thus a reporter of Shh signaling activity. In these embryos, we found reduced levels of Ptc-lacZ in the developing brain at both E11.5 and E14.5 (Figure 5B, 5D; Figure S5; data not shown). We also noted decreased expression of another Shh target gene, Gli1, in the retina and brain of *rudolph* mutants at E14.5 (Figure 5F, Figure S5). Furthermore, analysis using quantitative RT-PCR demonstrated reduced Ptc mRNA in

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**Figure 4. Sterol profiles.** Brain (A,B) and liver (C,D) tissue from wild-type (A,C) and *rudolph* (B,D) E14.5 embryos were used to create sterol profiles by gas chromatography-mass spectrometry. Some compounds identified include lanosterol (a), zymosterone (b), 4α-methyl-zymosterone (c), desmosterol (d), lathosterol (e), cholesterol (f), 4α-methyl-5α-cholest-8-en-3β-ol (g), 4α-methyl-5α-cholest-7-en-3β-ol and 4α-methyl-cholesta-8(9),24-dien-3β-ol (h), 4,4α-dimethyl-5α-cholest-8-en-3β-ol (i), 4,4-dimethyl-5α-cholesta-8(9),24-dien-3β-ol (j), and methylcholest-7-en-3-one (tentative) (k).

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Table 1. Analysis of sterols present in brain tissue.

| Substrates of Hsd17b7          | E14.5 Brain wt | E14.5 Brain rud |
|--------------------------------|----------------|----------------|
| Lanosterol (a)                 | 0.19           | 0.30           |
| 4α-methyl-5α-cholest-8-en-3β-ol (g) | 0.13           | 5.86           |
| 4α-methyl-5α-cholest-7-en-3β-ol (h) | 0.04           | 0.58           |
| 4α-methylcholesta-8(9),24-dien-3β-ol | 0.31           | 1.95           |
| 4,4’-dimethyl-5α-cholest-8-en-3β-ol (i) | 0.03           | 0.44           |
| 4,4’-dimethyl-5α-cholest-8(9),24-dien-3β-ol (j) | 0.49           | 2.16           |
| methylcholest-7-en-3-one (k)*   | 0.000          | 7.164          |

Sterols levels are quantified as percent of total sterols in tissue. Values from heterozygous embryos were very similar to those found in wild-type.

*, Tentative identification.

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Hedgehog signaling is disrupted in the **rudolph** mutant skeleton

We also observed limb patterning defects in mice from a mixed A/J, FVB, B6 (Figure 6A, 6B, Table S2). As **Hh** signaling is important for proper patterning, we examined **Hh** signaling in the developing limb bud. Normal patterning of the limb results in elevated Sbh signaling in the posterior portion of the developing limb bud as compared to the anterior. We observed Ptc expression in **rudolph** mutants (n = 3) from a mixed background using both Ptc-lacZ expression and whole mount in situ hybridization and found one embryo with reduced Ptc expression in the posterior limb bud (Figure 6D) as compared to littermate control (Figure 6C). The variable penetrance of the limb patterning phenotype is consistent with an incompletely penetrant reduction in Sbh activity in the developing limb bud. **Hh** signaling is also involved in long bone growth (where the relevant ligand is Indian hedgehog). We also see reduced expression of Ptc in the developing limb of **rudolph** mutants using either the Ptc-lacZ allele (Figure 6F) or an in situ riboprobe for Ptc (Figure 6H).

Hedgehog signaling is reduced in vitro upon reduction of Hsd17b7

To further determine if Hsd17b7 expression affects SHH signaling, we generated primary mouse embryonic fibroblasts (MEFs) from wild-type and **rudolph** embryos and assessed their response to added SHH protein. In wild-type MEFs, treatment with SHH protein resulted in increased cell proliferation and increased Ptc and Gli mRNA levels, which is consistent with the known role of SHH as a mitogen in several systems and with Ptc and Gli being direct targets of SHH signaling. In contrast, the effects of SHH treatment were blunted in mutant cells (Figure 7A–7C). We also generated MEFs from wild-type; Ptc-lacZ, and **rudolph**; Ptc-lacZ, embryos to measure SHH transcriptional activity via the accumulation of β-galactosidase. In this assay, wild-type; Ptc-lacZ MEFs responded to SHH treatment with increased β-galactosidase production, whereas **rudolph**; Ptc-lacZ MEFs did not (Figure 7D). Together these data suggest that **rudolph** mutant mice have reduced intracellular signal transduction distal to the binding of SHH ligand in the SHH signaling cascade.

In a parallel approach, we used Pzp53MED cells [20], which are SHH-responsive cells carrying the Ptc-lacZ allele, to assess the role of Hsd17b7 in **Hh** signal transduction. We used lentiviral infection followed by clonal selection with plasmids for RNAi knockdown of Hsd17b7 to create cell lines with reduced levels of Hsd17b7 (approx. 80% reduced, data not shown) and then treated the lines with recombinant SHH, as done with the MEFs. Two independent RNAi clones did not induce significant β-galactosidase production upon SHH treatment while the control cell line responded robustly (Figure 7E).

**Smoothened localization to the primary cilium is unaffected in the **rudolph** mutants

The decreased response to SHH protein in vitro shows that the signaling defect is downstream of ligand binding to cell surface receptors. Recent data have shown that treatment with oxysterols can cause a change in the subcellular localization of PTC and SMO protein [8], which is necessary but not sufficient for activation of the SMO protein [6,21]. The Hsd17b7 phenotype may be caused by dysregulated sterol biosynthesis affecting the localization and/or active state of the Smo receptor. We therefore examined the localization of a SMO-GFP fusion protein in wild-type and **rudolph** MEFs upon treatment with SHH, but found no decreased mobility of the SMO-GFP to the cilia in the mutant MEFs (Figure 8). Wild-type MEFs without SHH treatment had SMO-GFP throughout the primary cilium in only a subset of cells examined (43.3%; n = 29/67 ciliated, transfected cells in two independent experiments). Upon addition of SHH, SMO-GFP was found throughout the cilium in the majority of cells examined (89.6%; 65/73 cells). Mutant MEFs behaved similarly, with untreated cells having 38.7% SMO-GFP positive cilia (24/62) and SHH treatment leading to 86.7% (72/83) of cells with SMO-GFP throughout the cilium. In addition to the increase in cells with SMO-GFP throughout the cilium upon **SHH** treatment, we also note that untreated cells often had SMO-GFP largely at the base of cilium. **SHH** treatment resulted in very few cells showing SMO-GFP localization to the base of the cilium, but rather throughout the length of the cilium. Taken together, these data suggest that the **rudolph** mutation does not affect the localization of SMO within the primary cilium in response to **SHH** treatment.

**Discussion**

In this report, we describe the **rudolph** mouse mutant phenotype, which is caused by a mutation in the cholesterol biosynthetic enzyme Hsd17b7, the 3-ketosteroidreductase element of the sterol-4-demethylase complex. The **rudolph** phenotype includes marked abnormalities in the development of the nervous system and appendicular skeleton, which correlate with a decreased effectiveness of SHH signaling both in vivo and in vitro. Because sterols have...
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Ptc-lacZ

C

D

Gli1

F
been shown to affect SMO subcellular localization, we tested the movement of SMO to the primary cilium in response to SHH protein and found no defect in SMO trafficking. This suggests that the abnormality in rudolph affects the activation of SMO, resulting in a decreased response to SHH ligand binding.

**Sterols in rudolph mutants**

Our study of the sterols present in brain tissues from the mutant mice is consistent with reduced Hsd17b7 enzymatic function, as we observe an increase in compounds of the cholesterol biosynthetic pathway upstream of the Hsd17b7 enzyme. While no patient has yet been identified with a defect in Hsd17b7, increased levels of mono and dimethyl steroids have been reported in plasma and/or skin of patients with mutations in two other genes of the sterol-4-demethylase complex, SCAMOL (sterol C4-methyloxidase like) and NSDHL (NADPH steroid dehydrogenase-like protein) [22,23].

To explain the phenotype of the rudolph mouse we considered several possible metabolic effects, including 1) a decrease in the cellular level of cholesterol, 2) a decreased level of another product of Hsd17b7 enzymatic function, and 3) teratogenic effects of high levels of the cholesterol precursors detected in our study. Several lines of evidence suggest the last to be the major cause of the phenotype we observe. If simply a deficiency of the end-product, cholesterol, caused the rudolph phenotype, one might expect mice carrying mutations in the cholesterol biosynthetic pathway to resemble each other. This is not the case, since, despite some phenotypic overlap in adjacent disorders in the pathway, overall there are significant phenotypic differences across the spectrum of mouse models of human cholesterol biosynthetic disorders [24]. The best-characterized disorder of cholesterol biosynthesis is Smith-Lemli-Opitz syndrome [25,26], caused by mutations in DHCR7, which encodes the 7-dehydrocholesterol reductase that converts 7-dehydrocholesterol to cholesterol [15,27,28]. Two mouse models with null alleles of Dhcr7 have abnormal phenotypes including cleft palate, but lack the striking brain phenotypes we found in the rudolph mutant [29,30]. The Dhcr7 mutant mice have decreased cholesterol and increased 7-dehydrocholesterol levels in serum and tissues [29]. The enzyme immediately preceding DHCR7 is SC5DL (sterol C5-desaturase-like) which is deficient in human patients with lathosterolosis [31]. A null Sc5d allele in the mouse is a neonatal lethal with craniofacial and limb defects and decreased cholesterol similar to those of the Dhcr7-deficient mouse, but with increased levels of lathosterol (the substrate of Sc5d) in all tissues [32]. Given that the Dhcr7 and Sc5d mouse models have a significant decrease in cholesterol levels, which is not apparent in the rudolph mutant, and that their phenotypes do not resemble the rudolph phenotype, we conclude that cholesterol deficiency does not cause the distinctive embryological abnormalities of the rudolph mouse.

Nsdhl, another element of the sterol-4-demethylase complex, is the enzyme immediately preceding Hsd17b7 in the canonical cholesterol biosynthetic pathway [33]. NSDHL mutations in humans cause CHILD syndrome (congenital hemidysplasia with ichthyosiform erythroderma and limb defects), a rare X-linked dominant disorder with presumed lethality for CHILD causing alleles [34] and, with hypomorphic NSDHL mutations, CK syndrome, a form of X-linked mental retardation [22]. Mutations in Nsdhl are found in the Base patches (Bpa) and Striated (St) mice [35]. Analysis of tissue samples from Bpa/St females showed an accumulation of 4-methyl and 4,4′-dimethyl sterol intermediates. Human mutations in EBP cause X-linked dominant chondrodysplasia punctata (CDPX2, Conrad-Hunermann syndrome [36]). Patients with CDPX2 usually have normal plasma total cholesterol levels but increased levels of other sterols, including 8-dehydrocholesterol and cholesta-8(9)-en-3β-ol [36,37,38,39]. Tethered (Td) carries a missense mutation in the Ebp gene and phenotypically resembles the rudolph mutation most among all the known cholesterol biosynthesis mouse mutants. Male Td embryos die between E12.5 and birth and have defects in the skeleton and brain similar to those we describe here [40]. The sterol profile of heterozygous female mice includes elevated 8-dehydrocholesterol and cholest-8(9)-en-3β-ol. All of these findings combined with our data suggest a model in which the accumulation of specific sterols leads to the defects we observe. One of the effects these inhibitory sterols may be to dampen the intracellular response to Shh signaling.

**Rudolph mutants show similarities to other loss-of-function Shh phenotypes**

Although rudolph mutants lack some of the classic features of the Shh null mice, such as holoprosencephaly, other more specific ablations of SHH signaling have some features resembling the rudolph phenotype. In particular, the skeletal defects we observe in the rudolph mutant are similar to those described in the Indian hedgehog (Ihh) and dispatched-1 (Displ) loss of function mice and the conditional ablation of Smo from the developing skeleton [41,42,43]. As Ihh is the most active Ih ligand in development of long bones, we suggest that the similarities between rud and the HH-signaling mutants reflect the conservation of intracellular signaling transduction mechanisms between the different Ih ligands, and that these defects are due to an insufficient response to secreted IHH in the cartilage. In addition, the disorganized retina in rud mutants also resembles that seen in embryos with an ablation of Shh using a Thy1-Cre [44]. The role of Shh and Ihh as mitogens in retinal neuroblast proliferation has been established [45], and Shh, Ihh and Gli1 are expressed in retina at E12 and E13 [44,46]. The difference in timing between the cortical and retinal defects (Shh retinal molecular defects are seen at E14.5, but not E12.5, Figure S4) is consistent with the later expression pattern of Shh signaling components in the retina as compared to forebrain tissue.

The developing rudolph forebrain phenotype has both similarities to and differences from the known effects of Shh loss of function. The decreased cell proliferation and increased apoptosis we find are completely consistent with a role for Shh as a mitogen for the developing neural tissue and with the observations that blocking SHH function can lead to cell death [47], and that conditional ablation of Smo throughout the cortex by E9 using the Foxg1-Cre leads to increased cell death [48]. Emx1-Cre ablations of Shh and Smo in the dorsal telencephalon by E10 cause a smaller telencephalon featuring reduced proliferation and neuronal differentiation with increased cell death [49]. However, the
Figure 6. Sonic hedgehog signal transduction is abnormal in rudolph skeletal elements. (A,B) Embryos from a mixed background show an incompletely penetrant limb patterning defect. Whole mount in situ hybridization shows a Ptc domain in the posterior portion of the developing wild-type limb bud (C), which can be lost in rudolph mutants (D). Hedgehog signaling is also decreased in the developing long bones of rudolph mutants (F,H) compared to wild-type (E,G) as shown by Ptc-lacZ (E,F) or section in situ hybridization (G,H).

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striking disorganization of the cortex in rudolph mutants resembles more a loss of polarity phenotype, such as the cortical ablation of numb and numb-like [50]. Because Sbh loss of function has not been demonstrated to directly affect polarity, we suggest that abnormalities of cortical signaling mechanisms other than Sbh must be disrupted in the rudolph cortex to explain some of the developmental abnormalities of the CNS we observe. Because cortical dysplasia is not characteristic of any of the known human disorders of cholesterol biosynthesis, the extremely high CNS level of zymosterone, normally only a trace sterol in the brain, suggests that zymosterone or other 3-ketosterols in the rud brain could have a direct toxic effect or could otherwise impair neuronal differentiation.

**Rudolph is a hypomorphic allele of Hsd17b7**

Two reports have recently described the phenotypes of Hsd17b7 null allele mice [18,19]. These embryos have major morphological abnormalities by E10.5, precluding direct comparison to the

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**Figure 7. Sonic hedgehog signal transduction is abnormal in rudolph cells.** In vitro analysis also shows a decreased response to hedgehog protein in mouse embryonic fibroblasts (MEFs). Wild-type, but not mutant, MEFs show increased cell growth upon treatment with SHH (A; *p = 0.001 for wt; n = 4). Quantitative RT-PCR analysis also shows a reduced response of Gli1 (B; *p = 0.002 for wt, p = 0.01 for rud; n = 3) and Patched (C; *p = 0.001 for wt; n = 3) upon SHH treatment in mutant MEFs. β-galactosidase accumulates in SHH-treated wild-type Ptc-lacZ MEFs, but not rud Ptc-lacZ MEFs (D; *p = 0.005 for wt; n = 4). (E) PzP53Med (Ptc-lacZ;p53 medulloblastoma) cells infected and clonally selected with control pKO.1 lentivirus respond to SHH treatment with accumulation of β-galactosidase (* p = 0.07 for wt; n = 4). Two independent clonal lines infected with Hsd17b7 RNAi constructs do not respond as robustly to SHH treatment.

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Materials and Methods

Mouse husbandry and genotyping

Rudolph mice were originally generated by ENU mutagenesis of A/J mice and then outcrossed to FVB/J mice (both obtained from Jackson Labs, Bar Harbor, ME). Initial mapping was done with a whole-genome SNP panel similar to one we previously described [17], and the mutation mapped to a 19.6 Mb interval on chromosome 1. Exon directed sequencing (including some flanking intronic sequence to identify mutations potentially affecting splicing) identified the rudolph mutation. Genotyping is done with either D1Mit454 or D1Mit524 microsatellite markers depending on strains involved. We maintained the colony with a combination of intercrossing and outcrossing to FVB. The C57BL/6J Ptc1-lacZ mouse was obtained from the Jackson laboratory and intercrossed with rud heterozygous mice; Ptc1-lacZ genotyping was done with standard lacZ primers. We have also performed backcrosses of the rud allele to mice on C57BL/6J and 129X1/SvJ backgrounds. All animals were housed in accordance with the Harvard Medical School ARCM regulations. Timed matings were checked for signs of copulation in the morning; vaginal plugs were noted and noon of that day was established as embryonic day (E) 0.5.

Histology and immunohistochemistry

Embryos used for histological analysis were fixed with Bouin’s fixative for at least forty-eight hours and processed for paraffin embedding using a Leica TP1020 automated tissue processor. Sections were cut at a thickness of 14 μm and stained with hematoxylin and eosin using standard techniques. Microscopy was done with a Leica DC500 or Zeiss AxioImager with ApoTome. TUNEL assay was performed with the In Situ Cell Detection Kit, TMR Red, following the manufacturer’s instructions (Roche). BrdU labeling was done with a BrdU Labeling and Injection Kit (Roche). The TuJ1 antibody (SIGMA) was used at 1:500 for 2 hours at room temperature on paraffin sections with citrate buffer antigen retrieval. Neural tube immunohistochemistry was performed using standard methods with antibodies from the Developmental Studies Hybridoma Bank.

Skeletal measurements

To measure the size of the skeletal elements, embryos were stained for cartilage and bone using standard methods [57] and photographed. The length of each element was calculated using NIH ImageJ software, and units were converted to mm using standards.

In vitro analysis of SHH signaling

Mouse embryonic fibroblasts were generated using standard methods and plated at a density of 20,000 cells/cm² in the presence or absence of 200 ng/mL SHH amino terminal peptide (R&D Systems). Cell number was determined with the CyQuant Biosystems). Assays were performed 48 hours after plating. Cell growth experiments were done with an initial culture of 6,000 cells in a 96-well plate.
RNAi clones
Lentiviral particles were made via transfection of 293T cells with plasmids including a pKO.1 control and a validated RNAi construct against mouse Hsd17b7 (Open Biosystems, Huntsville, AL; clone TRCN0000041646). PZ53Med cells [20] were infected with 293T supernatant containing lentivirus. After puromycin selection, resistant cells were plated at clonal density and individual clones were isolated, maintained and analyzed with qRT-PCR for Hsd17b7 levels. Control and knock-down clones were treated with SHH protein as described above.

RT-PCR analysis
Total RNA from either brains or MEF cultures was prepared with TRIzol (Invitrogen) and cDNA was made with qScript cDNA synthesis kit (Quanta) or the SuperScript RTIII system (Invitrogen). Hsd17b7 transcripts were analyzed with both random hexamer primed cDNA and gene specific primed cDNA synthesis (primer: TTTTGGTACCTCAGCTCGGGTGACCGATTCTG). Hsd17b7 transcripts were analyzed with primers amplifying exons 6–8 (F: TCTGTATTCAGTGTTGTGTCR; G: TTTGGCCTGACGTGTTAAT; 259 bp) or exons 6–7 (F: TCTGTATTCAGTGTTGTGTCR; G: CACCAATTAGGGTAGAGCAA; 100 bp). Quantitative RT-PCR was done on a BioRad iCycler using either total RNA with Taqman probes (Applied BioSystems) or cDNA with Perfecta SYBR Green BioRad iCycler using either total RNA with Taqman probes (Applied BioSystems) or cDNA with Perfecta SYBR Green (Roche) following manufacturer's instructions. 48 hours after transfection, cells were fixed with 4% paraformaldehyde and Goat anti-mouse Alexa Flour 594 (Invitrogen) at 1:500 for 30 minutes at room temperature. Cells were mounted with VectaShield (Vector Laboratories) and imaged on a Zeiss AxioImager.

Sterol analysis
Sterols were extracted from brain tissue as previously described with the addition of sterol-specific ions for the compounds of interest to this study [61]. Sterol levels are reported as a fraction of total sterols.

Supporting Information

Figure S1 Variable Phenotypes of the Rudolph Mutant. In many mutants, the precursor to the nasal blood spot seen in late embryogenesis can be seen as a blebbing of the nasal epithelium as early as E12.5 (A). Introduction of the B6 genetic background to the colony resulted in some animals having more severe blebbing, which spreads beyond the tip of the nose (E18.5 in B, E12.5 in C). (A) The effect of the rudolph mutation on the splicing pattern of the Hsd17b7 cDNA (PCR reaction spanning exons 6–8) does not change among tissues (heart/hung, limbs, or brain) from wild-type (w), heterozygous (rud/+; b) or mutant (rud/rud; m), cDNA obtained from embryos on the C57BL6 background (whole embryo: B6) or from B6 brain shows the same pattern of splicing. (TIF)

Figure S2 Measurements of the long bones in both the forelimbs and hindlimbs at E16.5 and E18.5 show that mutant appendicular skeletal elements are significantly shorter at both stages. (TIF)

Figure S3 Rudolph mutants have phenotypes throughout the CNS. Disorganized neural tissue is seen in the neural tube (B) at E16.5. Analysis of the forebrain at E12.5 shows grossly normal organization of the mutant brain (D) but some disorganization of cortical tissue is apparent (F). (TIF)

Figure S4 The rudolph mutant form of Hsd17b7 protein is unstable. NIH3T3 cells were transfected with either wild-type Hsd17b7-GFP plasmid or a construct lacking the seventh exon to mimic the rudolph mutation (rud-GFP). Co-transfection with a Secb1-bmCherry plasmid serves to identify the endoplasmic reticulum (ER). Wild-type Hsd17b7-GFP is found in the ER as expected (A–C). Expression of the rud-GFP results in little or no GFP expression (D–F). (TIF)

Figure S5 Sonic hedgehog signaling in rudolph mutants. Patched-lacZ expression in rudolph;Ptc-lacZ brain (B) is weaker at E14.5 than in wild-type;Ptc-lacZ embryos (A). Gli1 expression in developing brain is also weaker in mutants (D) as compared to wild-type (C). (TIF)

Figure S6 Neural tube patterning is normal in rudolph mutants. Immunohistochemical analysis in the neural tube at E10.5 (A–N) and E12.5 (O–BB) for a variety of cell fates along the dorsal-ventral axis show no changes in patterning between wild-type and rudolph mutant embryos. (TIF)
Table S1  Long bone and digit length. Skeletal preparations of wild-type and radioshift embryos were prepared and the length of the skeletal elements were measured in Image J and converted to mm. (DOC)

Table S2  Mutant and phenotype incidence. Embryos were harvested from the genetic backgrounds indicated and associated phenotypes quantified. (DOC)

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Conceived and designed the experiments: RWS DRB. Performed the experiments: RWS AT-D PT LEK. Analyzed the data: RWS PT LEK

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