Non-cell autonomous inhibition of the Shh pathway due to impaired cholesterol biosynthesis requires Ptch1/2

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Abstract

Birth defects due to congenital errors in enzymes involved cholesterol synthesis like Smith-Lemli-Opitz syndrome (SLOS) and Lathosterolosis cause an accumulation of cholesterol precursors and a deficit in cholesterol. The phenotype of both SLOS and Lathosterolosis have similarities to syndromes associated with abnormal Sonic hedgehog (Shh) signaling, consistent with the notion that impaired cholesterol signaling can cause reduced Shh signaling. Two multipass membrane proteins play central roles in Shh signal transduction, the putative Resistance, Nodulation and Division (RND) antiporters Ptch1 and Ptch2, and the G-protein coupled receptor Smoothened (Smo). Sterols have been suggested as cargo for Ptch1, while Smo activity can affected both positively and negatively by steroidal molecules. We demonstrate that mESCs mutant for *7-dehydroxycholesterol reductase* (*7dhcr*) or *sterol-C5-desaturase* (*sc5d*) reduce the Hh response in nearby wildtype cells when grown in mosaic organoids. This non-cell autonomous inhibitory activity of the mutant cells required the presence of both Ptch1 and Ptch2. These observations support a model in which late cholesterol precursors that accumulate in cells lacking 7DHCR are the cargo for Ptch1 and Ptch2 activity that mediates the non-cell autonomous inhibition of Smo.

Introduction

Hedgehog (Hh) signaling is critically important during embryonic development and patterning in most animals. Initial events in the transduction of the Hh signal include binding of the ligand to the 12-transmembrane hedgehog receptor Patched 1 (Ptch1) which releases a Ptch1-dependent inhibition of the putative G-protein coupled receptor Smoothened (Smo) (Izzi et al., 2011; Marigo, Davey, Zuo, Cunningham, & Tabin, 1996; Taipale, Cooper, Maiti, & Beachy, 2002). In the absence of Shh, Ptch1 inhibits Smo non-stoichiometrically via a mechanism that remains poorly understood (Taipale et al., 2002).

Vertebrates have two Ptch homologs, Ptch1 and Ptch2 (Ptch1/2) that share overlapping functions (Alfaro, Roberts, Kwong, Bijlsma, & Roelink, 2014; B. Roberts, Casillas, Alfaro, Jägers, & Roelink, 2016). Ptch1/2 are members of the Resistance-Nodulation Division (RND) family of antiporters that are present in all domains of life. RND antiporters export their cargo in exchange for protons (Tseng et al., 1999) although it is possible that Ptch1/2 can utilize a sodium gradient (Myers, Neahring, Zhang, Roberts, & Beachy, 2017). In bacteria, RND transporters pump small lipophilic and amphiphilic molecules to the outside and eukaryotic RNDs could have a similar function.

Although the nature of the cargo of Ptch1/2 antiporter activity remains unknown, several observations suggest that it is a steroidal molecule (Hausmann, Mering, & Basler, 2009). The secretion of vitamin D/calcitriol and its precursor, vitamin D3/cholecalciferol, both derivatives of the cholesterol precursor 7-Dehydrocholesterol (7DHC), is Ptch1-dependent and these sterols repress Smo activity (Bijlsma et al., 2006; Linder et al., 2015). Several known Smo modulators have a steroidal backbone, including the steroidal alkaloid cyclopamine (Incardona, Gaffield, Kapur, & Roelink, 1998; Keeler, 1969; H. J. Sharpe, Wang, Hannoush, & de Sauvage, 2015).

All animal sterols are derived from lanosterol, which is modified in a series of enzymatic reactions in the postsqualene pathway, culminating in the production of cholesterol or vitamin D (Bloch, 1965; Chojnacki & Dallner, 1988; Ernster & Dallner, 1995; Takeyama et al., 1997).

Several congenital syndromes are caused by the loss of enzymes catalyzin the last steps in the cholesterol synthesis pathway. Smith-Lemli-Opitz (SLO) is caused by mutations in the gene encoding the 3β-hydroxysterol Δ7-reductase (7DHCR) (Fitzky et al., 1998), which catalyzes the

reduction of the 7 double bond in 7-Dehydrodesmosterol (Bloch, 1965; KANDUTSCH & RUSSELL, 1960). In SLO, a distinctive facial appearance including cleft palate, microcephaly, a small upturned nose, micrognathia, and ptosis is common. Furthermore, limb malformations like postaxial polydactyly, syndactyly of the second and third toe, and proximally placed thumbs are observed in most patients. This syndrome has some overlap with the malformations caused by reduced Shh signaling (SMITH, LEMLI, & Opitz, 1964). Biochemically, elevated levels of 7DHC and its isomer, 8-Dehydrocholesterol (8DHC), are found besides decreased cholesterol in serum and tissue of SLOS patients as well as in the mouse model (Tint et al., 1994; Wassif et al., 2001) . 7DHC levels are used for diagnosis SLO and are typically increased more than 50-fold (Abuelo et al., 1995).

Lathosterolosis is caused by mutations in the gene encoding the 3β-hydroxysteroid-Δ5-desaturase (SC5D) which desaturates lathosterol to 7DHC during cholesterol synthesis (Brunetti-Pierri et al., 2002). An additional role in the desaturation of cholesta-8(9)-en-3ß-ol to 8DHC is speculated (F. D. Porter, 2002). Symptoms of lathosterolosis have similarities with those observed in SLOS, including postaxial polydactyly, microcephaly, micrognathia, and toe syndactyly (Brunetti-Pierri et al., 2002). In the lathosterolosis mouse model, lathosterol levels are elevated 60 fold whereas levels of cholesterol were decreased similar to the SLOS mouse model (Blassberg, Macrae, Briscoe, & Jacob, 2016; Krakowiak et al., 2003; Wassif et al., 2001). Circulating maternal cholesterol can be transported to the fetus, making it unlikely that SLOS and lathosterolosis are predominantly caused by low fetal cholesterol, but instead it is plausible that the accumulation of late sterol precursors contributes to these birth defects, possibly by suppressing Shh signaling (Incardona et al., 1998).

Cell types in the developing neural tube arise in at stereotypic position in response to Shh signaling. An effective in vitro model of the neural tube are neuralized embryoid bodies (nEBs), also called spinal cord organoids (SCOs), which are aggregates of approximately 10^4 embryonic stem cells (ESCs) (Wichterle, Lieberam, Porter, & Jessell, 2002). Mosaic nEBs can be generated by mixing cells with genotypically diverse cell lines in varying ratios. This approach allows us to address the non-cell autonomous effects caused by genetic alterations.

Here, we provide evidence that accumulation of late sterol precursors can inhibit Shh signaling both cell-autonomously, as well as non-cell autonomously even in cells with an intact cholesterol synthesis pathway. Importantly, this increased non-cell autonomous inhibition requires the presence of Ptch1/2 function in those cells that accumulate the cholesterol precursors.

These observations are consistent with a model that late sterol precursors are the cargo of Ptch1/2 antiporter activity in its ability to inhibit Smo non-cell autonomously.

Results

The loss of 7-Dehydrocholesterol reductase and/or Lathosterol 5-desaturase changes the cellular distribution of sterols

We have shown before that Ptch1/2 activity can inhibit Smo non-cell autonomously, supporting the notion that Ptch1/2 functions in the distribution or secretion of a Smo inhibitor (B. Roberts et al., 2016). To assess if this inhibitor could be a late sterol precursor, we made *Shh*$^{-/-}$*;7Dhcr*$^{-/-}$ cells, and found that these cells have reduced amounts of cholesterol after three days of serum starvation as compared to the parental *Shh*$^{-/-}$ cells (Figure 1). Similar to SLOS, the phenotype of lathosterolosis overlaps with that of congenital Shh malformations, raising the question if similar mechanisms impair the Shh signal transduction. To assess more global changes in the distribution sterols, we stained with Filipin, a fluorescent stain (Blanchette-Mackie, 2000) used in the diagnosis of Niemann-Pick disease type C (Sokol et al., 1988). Besides *Shh*$^{-/-}$*;7Dhcr*$^{-/-}$ cells, we also stained *Shh*$^{-/-}$*;Sc5d*$^{-/-}$ cells as well as the triple mutant *Shh*$^{-/-}$;*7Dhcr*$^{-/-}$;*Sc5d*$^{-/-}$ cells, cultured as SCOs in the absence of serum for up to 72 hours. Cells lacking only Shh had surface and wrinkle-like accumulations of sterols on the cell surface on the first and second day of serum deprivation (Figure 1). However, few cells appeared with small circular-shaped structures on the cell surface as well as inside the cell that accumulated on day 3. Whereas cells missing either 7Dhcr or Sc5d had a wild-type like distribution of sterols, *Shh*$^{-/-}$*;7Dhcr*$^{-/-}$;*Sc5d*$^{-/-}$ cells appeared to have a reduced amount of sterols. These results indicate that a lack of Sc5d is not epistatic to a lack of 7Dhcr, which are thought to act in sequence in the cholesterol synthesis pathway, but in fact has an additive effect.

Accumulation of late sterol precursors inhibit the Hh response

The loss of DHCR7 in SLOS is known to suppress Shh signaling, and the prevailing hypothesis is that this is caused by the accumulation of a late sterol precursor inhibiting Smo. To discern whether the accumulation of the direct cholesterol precursor 7DHC or the lack of cholesterol can inhibit Shh signaling, SCOs constituting of *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$*;Shh*$^{-/-}$ were treated with 13 µM 7DHC, 13 µM cholesterol, or a combination of both sterols, and the amount of *Ptch1:LacZ* was measured as a proxy for the Hh pathway response (Figure 1). The reporter cells are devoid of both Ptch1 and Ptch2 with the result that Smo is constitutively active and the *Ptch1:LacZ* expression levels are highly increased compared to heterozygous reporter cells (*Ptch1*$^{+/LacZ}$*;Shh*$^{-/-}$, Alfaro et al. 2014, Roberts et al. 2016). 7DHC significantly reduced the Shh pathway activity in *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$*;Shh*$^{-/-}$ SCOs by 30 %. Cholesterol had no effect on the Hh response, neither when added alone nor in combination with 7DHC, indicating that the accumulation of cholesterol precursors is the inhibitory event and not a lack of cholesterol and that additional cholesterol cannot compensate the inhibitory effect of 7DHC. These results suggest that the inhibitory cholesterol precursor can be found in the medium, and we therefore tested the inhibitory potential of conditioned supernatants. We transferred medium of SCOs consisting of *Ptch*$^{-/-}$ and/or *7Dhcr*$^{-/-}$ cells to *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$*;Shh*$^{-/-}$ reporter cells and found that in addition to a Ptch-mediated inhibition, the conditioned medium of *7Dhcr*$^{-/-}$ cells decreased the Hh response in reporter cells by 40% (Figure 1). These results support the idea that a cholesterol precursor can inhibit the Hh response non-cell autonomously.

Loss of 7-Dehydrocholesterol reductase and/or lathosterol 5-desaturase inhibit the Shh pathway non-cell autonomously

To test if endogenous early cholesterol precursors like 7DHC or lathosterol can inhibit Shh signaling non-cell autonomously, we measured *Ptch1:LacZ* expression in our *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$*;Shh*$^{-/}$ reporter cells in mosaic nEBs with 50% *Shh*$^{-/-}$*;7Dhcr*$^{-/-}$, *Shh*$^{-/-}$*;Sc5d*$^{-/-}$ or the triple mutant *Shh*$^{-/-}$*;7Dhcr*$^{-/-}$*;Sc5d*$^{-/-}$ mESCs. Both *Shh*$^{-/-}$*;7Dhcr*$^{-/-}$ and *Shh*$^{-/-}$*;Sc5d*$^{-/-}$ cells inhibited Shh pathway activity in the neighboring reporter cells (Figure 2). The inhibition by *Shh*$^{-/-}$*;7Dhcr*$^{-/-}$ cells, however,

was significantly more potent than the one caused by *Shh*$^{-/-}$;*Sc5d*$^{-/-}$ mESCs, suggesting that 7DHC could be a major component of the inhibitory cholesterol precursors. Interestingly, a combination of the two mutations further decreased the *Ptch1:LacZ* expression in SCOs composed of even amounts of *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$;*Shh*$^{-/-}$ and *Shh*$^{-/-}$;*7Dhcr*$^{-/-}$;*Sc5d*$^{-/-}$ mESCs. This additive effect, as mentioned earlier, contradicts the idea of a linear pathway with Sc5d and 7Dhcr being enzymes acting in sequence. The differences in the inhibitory potential of the different cell lines could not be explained by shifted ratios of reporter and repressor cells since the amount of CMFDA Green stained reporter cells remained the same in all 4 different types of mosaic nEBs 48 h after aggregation (Figure 2).

These results suggest that a variety of early cholesterol precursors can inhibit Shh signaling non-cell autonomously. Moreover, the additive inhibitory effect of *Shh*$^{-/-}$;*7Dhcr*$^{-/-}$;*Sc5d*$^{-/-}$ on the Shh pathway contradicts the linear cholesterol biosynthesis pathway of textbooks.

Ptch1/2 is required for non-cell autonomous inhibition

We tested if Ptch1/2 is required for non-cell autonomous inhibition by cells deficient for 7Dhcr or Sc5d and compared the response to 7DHC in homogenous *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$;*Shh*$^{-/-}$ reporter nEBs to mosaic nEBs consisting of 50% *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$;*Shh*$^{-/-}$ reporter and 50% Ptch-containing cells (Figure 3). Exogenous 7DHC decreased the Hh response in reporter cells to a much greater extend when Ptch1/2-containing cells were present, additionally to the inhibitory function of Ptch1/2 alone (both types of nEBs were normalized to their respective mock-treatment). This suggests that 7DHC from the medium can be acted upon and potentiated in its Smo-inhibitory potential by Ptch1/2. This effect of Ptch1/2 on 7DHC was not observed for the Smo antagonist cyclopamine, suggesting that cyclopamine inhibits Smo directly without the need for Ptch1/2 function (Figure S1).

We next sought to assess if Ptch1/2 function can be rescued and if both homologs contribute to Smo-inhibition. As overexpression experiments in mESCs usually suffer from poor transfection efficiencies, we overexpressed Ptch1 or Ptch2 in Ptch1/2- or 7Dhcr-deficient fibroblasts (*Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ and *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$;$^{7Dhcr-/-}$) and transferred their supernatant onto

Light2 reporter cells. Conditioned medium from Ptch1- and Ptch2-expressing cells inhibited the Shh pathway response in the presence of SAG equally and independent of 7Dhcr in the conditioning cells.

To further assess the role of Ptch1/2 in non-cell autonomous inhibition of Shh signaling by cholesterol precursors, we mixed Ptch1/2-deficient *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$*;Shh*$^{-/}$ and *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$*;Shh*$^{-/-}$*;7Dhcr7*$^{-/-}$ mESCs in mosaic nEBs and compared their *Ptch1:LacZ* expression to nEBs consisting of either genotype alone. While the Hh response is constitutively high in *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$*;Shh*$^{-/-}$ cells, the loss of 7Dhcr decreases Ptch1:LacZ expression by approximately 70 % (Figure 3). If Ptch1/2 are indeed required for non-cell autonomous Shh pathway inhibition, 7Dhcr- and Ptch1/2 deficient cells should not be able to inhibit the Shh pathway response and the *Ptch1:LacZ* expression of the mosaic nEBs should be exactly half of the difference between *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$*;Shh*$^{-/}$ and *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$*;Shh*$^{-/-}$*;7Dhcr*$^{-/-}$ nEBs. The null hypothesis would therefore be $H_0 = 1 – ½$ (average *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$*;Shh*$^{-/-}$- average *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$*;Shh*$^{-/-}$*;7Dhcr*$^{-/-}$), indicated by a red line in Figure 3A. Indeed, the average of Ptch1/2-deficient mosaic nEBs was only 2 percent points higher than H_0.

To further discern the role of Ptch1/2 in repressing and responding cells, we mixed 5% *Hb9:gfp* mESCs with a panel of Ptch1/2-containing/deficient and/or 7Dhcr-containing/deficient mESCs. The Hh pathway response was activated in the reporter cells with the Smoothened agonist SAG and the inhibitory potential of the interspersed cells was assessed by counting the number of *Hb9:gfp*-positive nuclei as a measure of Hh pathway activation. Compared to uninhibited *Hb9:gfp* reporter cells in Ptch1/2 -deficient mosaic nEBs, Ptch1/2 alone decreased the number of positive nuclei by more than half. Consistent with our results described earlier, Ptch1/2 containing but 7Dhcr deficient cells decreased the number of positive nuclei even further. In the absence of Ptch1/2 and 7Dhcr, however, Hb9:gfp expression was uninhibited, similar to the level of Ptch1/2-deficient mosaic nEBs. This suggests that a loss of Ptch1/2 is epistatic to a loss of 7Dhcr in repressing cells and that Ptch1/2 is required for non-cell autonomous inhibition. As the addition of SAG increased the number of *Hb9:gfp* positive nuclei in every condition, the inhibitory factor in the *7Dhcr*$^{-/-}$ cells is likely a competitive inhibitor to SAG, meaning that it acts at the level of Smo.

Discussion

Mutations in the *DHCR7* gene encoding the 3β-hydroxysteroid Δ^7-reductase are associated with the Smith-Lemli-Opitz Syndrome (SLOS), a congenital multiple malformation syndrome (Fitzky et al., 1998). Malformations resemble birth defects observed with reduced hedgehog signaling during embryogenesis (Roessler et al., 1996), raising the hypothesis that the accumulating cholesterol precursor 7DHC reduces Shh signaling. Here, *Shh*$^{-/-}$*;7Dhcr*$^{-/-}$ mESCs were found to inhibit the Shh pathway non-cell autonomously. Importantly, Ptch1/2 was found to enhance the inhibitory effect of exogenously added 7DHC on reporter cells devoid of Ptch1/2, arguing for 7DHC being an endogenous cargo of Ptch1/2. Furthermore, Ptch1/2 are required for non-cell autonomous inhibition as *7Dchr*$^{-/-}$ cells also deficient for Ptch1/2 were not able to inhibit the Hh response in adjacent cells.

Possible scenario for the Ptch1/2-dependent inhibition of Smo by cholesterol precursors

The following model based on the data presented here evolved (Figure 4): Ptch1/2 is able to inhibit Smo non-cell autonomously and its inhibitory function is even enhanced when the cells are deficient for one or more enzymes of the cholesterol synthesis pathway. This is likely due to the transport of inhibitory cholesterol precursors like 7DHC, increasing their inhibitory potential by a yet unknown mechanism (Bijlsma et al., 2006; Cortes et al., 2015; Linder et al., 2015). To this point, we cannot exclude that a derivative of 7DHC like vitamin D3/cholecalciferol or vitamin D/calcitriol inhibits Smo. To be inhibitory non-cell autonomously, 7DHC and other cholesterol precursors need Ptch1/2 activity, as Ptch1/2-deficient cells had no inhibitory potential, independent of the presence of 7Dhcr or Sc5d.

Does Ptch transport a Smo-inhibitory sterol?

Many Smo-modulating small molecules, both endogenous and exogenous as well as activating and inhibiting, are known. Some of those have a steroidal structure, which, together with evidence originating from Ptch, suggests that Ptch uses a sterol to inhibit Smo in the absence of the Hh ligand. Cyclopamine, despite its steroidal structure and inhibitory function on the Hh response, inhibits Smo via direct binding (Chen, Taipale, Cooper, & Beachy, 2002). We find that

Ptch1/2 does not exacerbate Smo inhibition by cyclopamine, arguing against it being a cargo of Ptch1/2. Cholesterol on the other hand would be a welcome endogenous candidate as a Ptch cargo due to its ubiquitous presence although definitive evidence, especially for a Smo inhibitory function, has not been found.

Besides cholesterol, vitamin D/calcitriol can be synthesized from 7DHC via photolysis. When 7DHC accumulates in the absence of 7Dhcr, the synthesis of vitamin D is increased (Prabhu, Luu, Sharpe, & Brown, 2016). Vitamin D3/cholecalciferol, the precursor of vitamin D, is capable of inhibiting Smo more efficiently than 7DHC and is secreted by Ptch1 (Bijlsma et al., 2006; Tang et al., 2011). Similarly, the hormonally active form of vitamin D3, calcitriol, was reported to be released into the extracellular space by Ptch1 and efficiently inhibit the translocation of Smo to the primary cilium (Cortes et al., 2015; Linder et al., 2015).

Evidence for alternative pathways in the cholesterol biosynthesis pathway

Cholesterol synthesis begins with acetyl-CoA and has a multiplicity of intermediates, each requiring catalyzation by unique enzymes to progress to the next precursor molecule. Kandutsch and Russell proposed a linear but bifurcated pathway in 1960 for the synthesis of cholesterol that only differ in an additional double bond in the alkyl side chain appended to the CD-ring. This double bond can be resolved at any time by the 3β-hydroxysterol Δ^{24}-reductase. Sc5d acts upstream of 7Dhcr and a mutation would therefore logically be epistatic to a mutation in the *7Dhcr* gene. Contrary, we observed an additive inhibitory effect of the two mutations in combination on the Shh pathway. Thus, additional functions of enzymes or additional, Sc5d and/or 7Dhcr circumventing pathways are conceivable. Porter et. al proposed, with reservation, that 8-Dehydrocholesterol (8DHC), an isomer of 7DHC, can be synthesized from the precursor of lathosterol (cholesta-8(9)-en-3ß-ol) by Sc5d (F. D. Porter, 2002). 8DHC and 7DHC differ in the position of a double bond that can be swapped in both directions by the enzyme that synthesizes lathosterol from its precursor, 3β-hydroxysterol Δ8,Δ7-isomerase (EBP). Although this alternative pathway does not explain the additive inhibitory effect of combinatorial Sc5d and 7Dhcr knock-outs - a lack of Sc5d would still terminate the synthesis of cholesterol at the level of lathosterol - it still strengthens the likelihood that cholesterol synthesis is not as linear as textbooks impart and that enzymes of cholesterol synthesis can exhibit additional, unknown functions.

The mechanism of Smo inhibition by Ptch1/2

The hypothesis that the non-cell autonomous inhibition of the Shh pathway by Ptch1/2 is mediated by its antiporter function originates from the observation that a mutation in the antiporter channel (Ptch1D499A) blocks Ptch1 activity and subsequently, the Hh response (Alfaro et al., 2014; Taipale et al., 2002).

As a member of the RND family of proton driven antiporters Ptch1/2 is thought to require a proton gradient to transport molecules across membranes (Tseng et al., 1999). Sodium has been proposed as an alternative ion that would be transported across the cell membrane in exchange for the Smo inhibitory molecule. Besides the question of "what" Ptch1/2 transports, the "how/where" has been the point of more recent discussion. The Ptch1 monomer revealed a hydrophobic pore-like structure in cryo-electron microscopy (cryo-EM) models, oftentimes with sterols in the vicinity of the pore opening. Transport of sterols through that pore, however, are difficult to reconcile with a simultaneous antiport of an ion through the seemingly narrow diameter of the pore. Dispatched, another member of the RND superfamily, is a homotrimer, suggesting that Ptch1, too, could form a trimer with a large opening in the center. This central pore could transport the Smo-inhibitory sterol, leaving the pores in the individual monomers to function as an ion channel.

Our findings suggest that 7DHC and other cholesterol precursors are endogenous cargos of Ptch1/2. Similar to other (bacterial) members of the RND transporter family, Ptch1/2 can mediate the efflux of substrates that inhibit Smo and thus the downstream transcriptional response.

Materials and Methods

CRISPR/Cas9 mediated mutagenesis

The CRISPR/Cas9 genome editing technique was used to knock out the *7Dhcr* and *Sc5d* gene (Ran et al. 2013). The pX458/ pSpCas9(BB)-2A-Puro and pX459/ pSpCas9(BB)-2A-GFP expression plasmids were a gift from the Doudna lab (University of California, Berkeley, USA). sgRNAs were designed using the online tool provided by the Zhang lab (http://tools.genome-engineering.org, MIT, Massachusetts, USA) and ordered from IDT DNA Technologies (Iowa, USA).

Table 1: sgRNAs used for knocking out the 7Dhcr and Sc5d gene. 20 nucleotide-target sequences are bolt. With the exception of the second exon of the 7Dhcr gene, the first two sgRNAs target an upstream sequence, the last two a downstream sequence. The second sgRNAs were designed as a backup in case of too low efficiency of the first one.

Target	top sgRNA	bottom sgRNA
7Dhcr exon 1 + 2	CACCGCTGTCGCTGTGATGAACACG	AAACCGTGTTCATCACAGCGACAGC
	CACCGAACACGTGGCAAAAAGCAGC	AAACGCTGCTTTTTGCCACGTGTTC
	CACCGAATGAACTACAATTCGGAAT	AAACATTCCGAATTGTAGTTCATTC
	CACCGGCTCACCAGTGACCCTTATC	AAACGATAAGGGTCACTGGTGAGCC
7Dhcr exon 2	CACCGGAAGTGGACTGGTTCTCCC	AAACGGGAGAACCAGTCCACTTCC
	CACCGACCCACAAGGCATACAGTT	AAACAACTGTATGCCTTGTGGGTC
Sc5d exon 6	CACCGCATGACTTGCAAACGGCGT	AAACACGCCGTTTGCAAGTCATGC
	CACCGCTCTGAAGGAAGCCGTCCAC	AAACGTGGACGGCTTCCTTCAGAGC
	CACCGACATTGTGGGATAGAATTGG	AAACCCAATTCTATCCCACAATGTC
	CACCGTTTGCAGAAAACGGCTGTAA	AAACTTACAGCCGTTTTCTGCAAAC

Top and bottom strands were annealed using the T4 PNK (New England Biolabs). The plasmids were digested using the FastDigest BpiI restriction enzyme (Thermo Fisher Scientific). sgRNAs targeting upstream sequences were ligated into the digested pX459 using the T4 DNA ligase (Thermo Fisher Scientific) whereas sgRNAs targeting downstream sequences were ligated into the digested pX458 plasmid. The plasmids were then transformed into competent DH5α bacteria using standard procedures and plated on LB agar plates containing 100 µg/ml carbenicillin. The next day, two colonies per plate were picked and transferred to 3 ml LB medium containing 100 µg/ml carbenicillin. A day later, the plasmids were isolated using the QIAprep spin miniprep kit (Qiagen) according to the manufacturer's instructions. Correct insertion of the sgRNA into the plasmid was checked by sequencing from the U6 promoter (primer sequence: GAGGGCCTATTTCCCATGATTCC).

The mESCs (*Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$;*Shh*$^{-/-}$ and *Shh*$^{-/-}$) were transfected in 6 well plates coated with gelatin using the recommended amounts of Lipofectamine2000 (Thermo Fisher Scientific) and DNA. An initial pre-selection with puromycin was done the day after transfection with 1 µg/ml puromycin for one day. Approximately one week after transfection, clonal colonies had grown and were checked for transfection with a Zeiss Observer fluorescence microscope. Green fluorescent colonies were picked under sterile conditions with a pipet tip and transferred to a 96 well plate coated with gelatin for expansion. Small amounts of cells were taken, heated at 95 °C, frozen, and taken for PCR amplification. PrimeStar® (Takara Clontech) PCR was done at the recommended parameters (10 µl DNA in 0.05 % trypsin, 9 µl 2X PrimeStar PCR buffer, 0.3 µl 10 mM primer, 0.3 µl 10mM dNTPs, 0.07 µl PrimeStar® HS DNA polymerase, denaturation at 98 °C for 10 s, annealing at 60 °C for 5 s, extension at 72 °C for 1 min, 35 cycles). 10 µl of the PCR product were analyzed on a 1.5 % agarose gel. Usually 3 – 5 positive clones without a detectable wild-type band were sent for sequencing to validate a deletion.

Table 2: PCR primers used for validation of a deletion.

Target	forward primer (5' → 3')	reverse primer (5' → 3')
7Dhcr exon 1 and 2	AGATCCTGCACAAAGCGCAC	TCAGTCTCACGCAGCAAGCAG
7Dhcr exon 2	AGCCCAAGGGATGGGACATTTC	TGTAACTGCTGCACCCTGACTTG
Sc5d exon 6	TCTAGCCGCCAGCCATTACA	CGGTGTCTTCCCGCTGTTTAC

Tissue culture

Ptch1$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$;*Shh*$^{-/-}$ and *Shh*$^{-/-}$ mESCs were generated in the Roelink lab using TALEN mediated mutagenesis and described earlier (Roberts et al., in preparation). The *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$;*Shh*$^{-/-}$ line originates from the *Ptch1*$^{LacZ/LacZ}$ line (Goodrich et al. 1997). All cell lines were cultured under standard conditions in tissue culture dishes coated with gelatin in ES medium (DMEM/Gibco, 15 % FBS/Gibco, 2mM L-glutamine/Thermo Fisher Scientific, 1X Penicillin Streptomycin/Thermo Fisher Scientific, 1X MEM Non-Essential Amino Acids Solution/Thermo Fisher Scientific, 1X Nucleosides for ES cells/EMD Millipore, 1X ß-Mercaptoethanol, 1X GST-

LIF/Millipore). Cells were typically grown to 80 – 90 % confluency and split 1:10 every other day using 0.05 % trypsin/Invitrogen.

For generation of nEBs, mESCs were singularized using 0.05 % trypsin/Invitrogen, washed twice with PBS, and counted in a Neubauer counting chamber. 2 x 10^5 – 4 x 10^5 cells were seeded in 60mm Petri dishes in DFNB medium (Neurobasal medium/Gibco, DMEM F12 1:1/Gibco, 1.5 mM L-glutamine/Thermo Fisher Scientific, 1X Penicillin Streptomycin/Thermo Fisher Scientific, 1X B27® Supplement/Gibco, 0.1 mM ß-Mercaptoethanol) and incubated for 48 h with agitation (0.8 Hz). After 48 h, 10 µM Retinoic Acid (Sigma/Aldrich) was added.

7DHC, cholesterol, and lathosterol (all Sigma/Aldrich) were dissolved in ethanol and used at 13 µM. All reagents were added directly to aggregating nEBs.

Cell tracking

For cell lineage tracking experiments, cells were incubated with 500 µM CMFDA Green or CMAC Blue (both Invitrogen) in DFNB medium for 45 min at 37 °C and seeded as described above. After 48 h (or respective time points), nEBs were fixed in 4 % paraformaldehyde (Thermo Fisher Scientific) in 1X PBS for 10 min and mounted directly in Fluoromount G Mounting Medium (Thermo Fisher Scientific). Images were taken with a Zeiss Observer fluorescence microscope with a 20X and 10X objective.

Filipin staining

For filipin staining, nEBs were fixed in 4 % paraformaldehyde (Thermo Fisher Scientific) in 1X PBS for 10 min after one, two, or three days. nEBs were stained in 30 µM filipin III (Sigma/Aldrich) in 1X PBS for 30 min in the dark and mounted in Fluoromount G Mounting Medium (Thermo Fisher Scientific) after washing once with 1X PBS. Images were taken with a Zeiss Observer fluorescence microscope with a 63X objective and oil immersion.

Reporter Gene Assay for *Ptch1:LacZ* induction

Ptch1:LacZ expression levels were quantified 72 h after aggregation of nEBS using the Galacto-Light Plus™ system (Applied Biosciences) according to the manufacturer's instructions. Shortly, nEBs were collected in plastic tubes, washed with 1X PBS, and lysed using lysis buffer (100 mM potassium phosphate pH 7.8, 0.2% Triton X-100). Lysates were incubated with 70 µl Reaction

buffer for 30 min in a 96 well plate, followed by an incubation with Accelerator-II for 15 min. Signal was read in a microplate luminometer for 5 s per well. *Ptch1:LacZ* levels were normalized to total protein using the Bradford reagent (BioRad).

Statistics

Triplicate samples were analyzed within each experiment and all data points were normalized. P-values were calculated using the Two-Sample t-Test assuming unequal variances in Microsoft Office Excel 2016 and considered significant <0.01. At least three independent experiments were analyzed.

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A

lathosterol

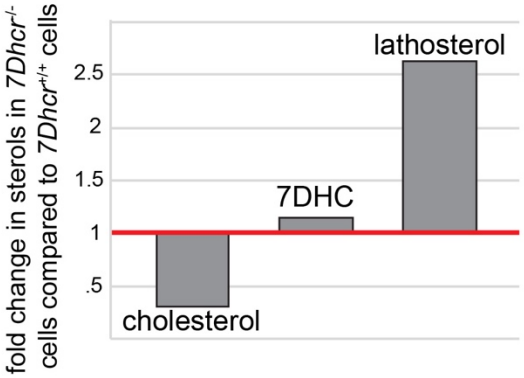
↓ Sc5d

7Dehydrocholesterol (7DHC)

↓ 7Dhcr

cholesterol

B



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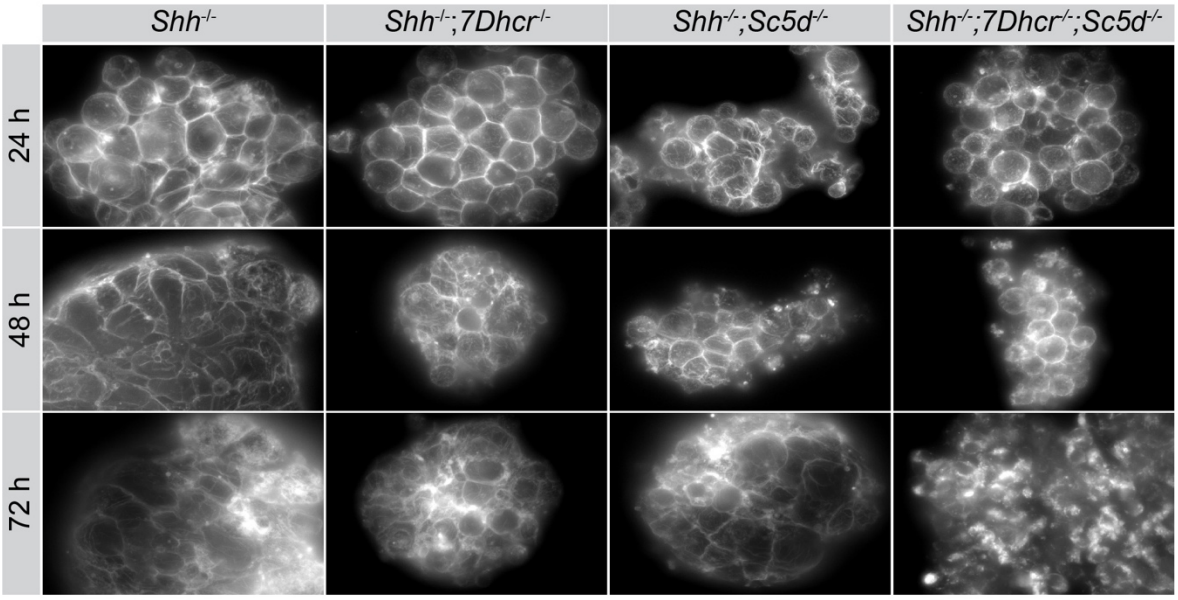

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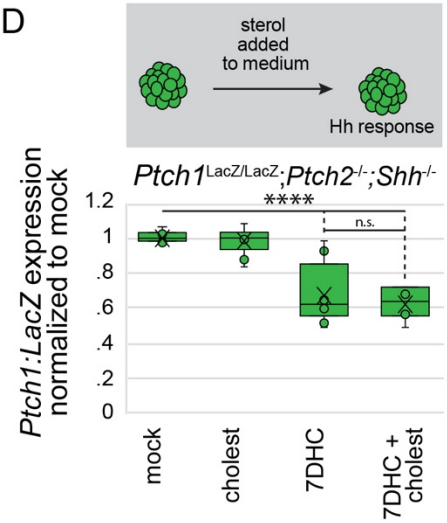

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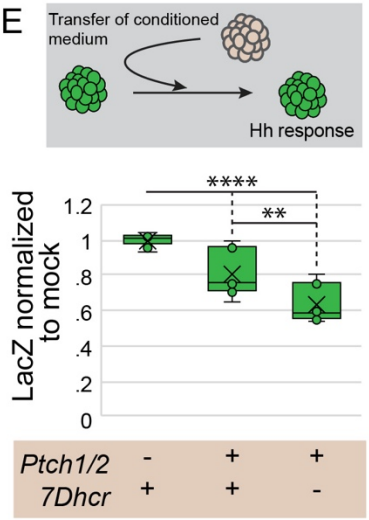


Figure 1: *Shh*$^{-/-}$;*7Dhcr*$^{-/-}$ mESCs inhibit the Shh pathway non-cell autonomously

(C) *Ptch1:LacZ* expression levels were measured in mosaic nEBs with *Ptch1*$^{LacZ;LacZ}$;*Ptch2*$^{-/-}$;*Shh*$^{-/-}$ and *Shh*$^{-/-}$ or *Shh*$^{-/-}$;*7Dhcr*$^{-/-}$ mESCs (ratio: 1:1) after 72 h of culture. Genotypes of the repressor cells are shown in boxes beneath the graph. Shown are means of 9 independent experiments +/- s.e.m.

(D) mESCs were stained with CMFDA Green (*Ptch1*$^{LacZ;LacZ}$;*Ptch2*$^{-/-}$;*Shh*$^{-/-}$) or CMAC Blue (*Shh*$^{-/-}$ or *Shh*$^{-/-}$;*7Dhcr*$^{-/-}$) cell tracker prior to aggregating to mosaic nEBs. Images were taken 48 h after aggregation. CMAC Blue dye was recolored in magenta. Genotypes are shown in boxes above and beneath the images in the respective color.

(E) *Ptch1*$^{LacZ;LacZ}$;*Ptch2*$^{-/-}$;*Shh*$^{-/-}$ nEBs and mosaic nEBs with either *Shh*$^{-/-}$ or *Shh*$^{-/-}$;*7Dhcr*$^{-/-}$ repressor cells were treated with either ethanol (mock), 13 µM cholesterol, 13 µM cholesterol + 13 µM 7DHC, 13 µM 7DHC starting at day 0 and their *Ptch1:LacZ* expression was measured after 72 h. Shown are means of at least three independent experiments +/- s.e.m. (cholesterol n = 3, cholesterol + 7DHC n = 3, 7DHC n = 4). ** $p < 0.001$; **** $p < 0.0001$; n.s. = not significant.

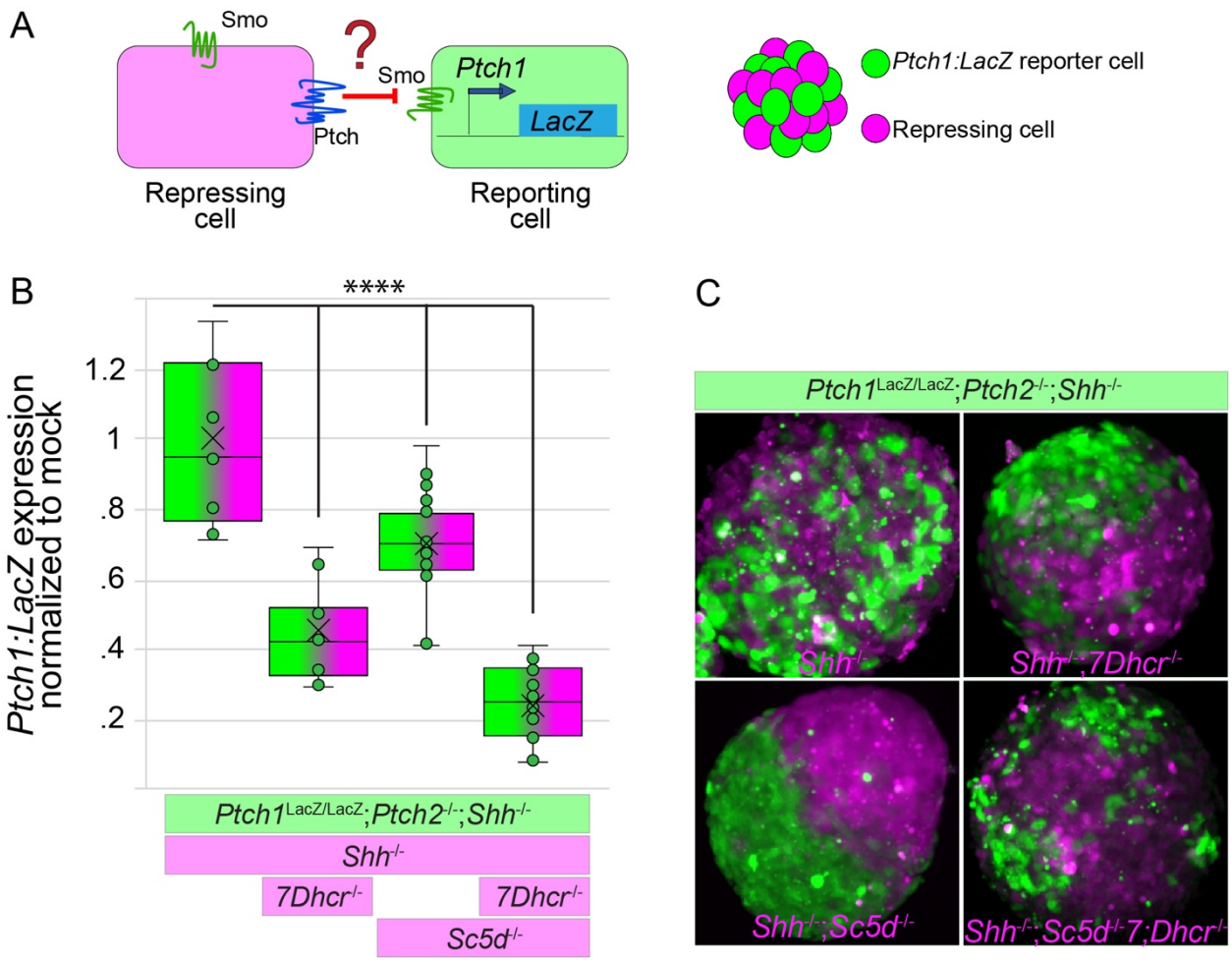
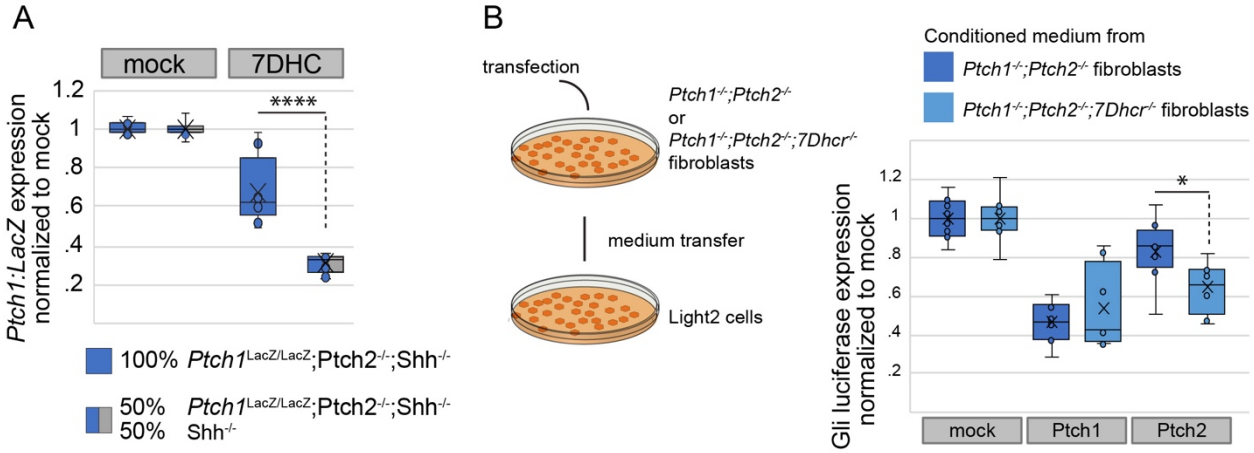


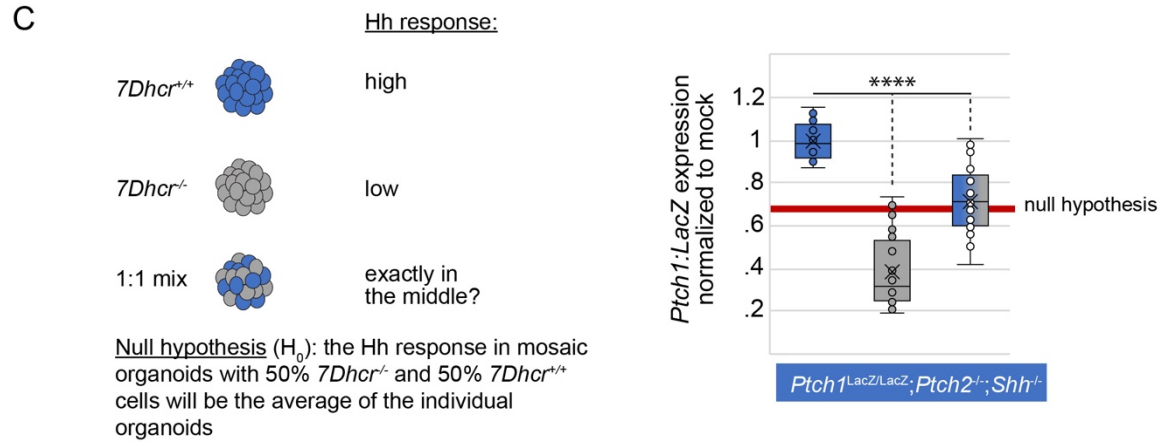
Figure 2: **Deletions in the Sc5d gene in mESCs inhibit the Shh pathway non-cell autonomously** similarly to *Shh*$^{-/-}$*;7Dhcr*$^{-/-}$ mESCs but are not epistatic.

(C) *Ptch1:LacZ* expression levels were quantified in *Ptch1*$^{LacZ;LacZ}$*;Ptch2*$^{-/-}$*;Shh*$^{-/-}$ mESCs which were cultured in mosaic nEBs (ratio: 1:1) with *Shh*$^{-/-}$ mESCs or *Shh*$^{-/-}$ cells with mutations in the *7Dhcr* and/or *Sc5d* gene for 72 h. Shown are means of 5 independent experiments with 2 *Shh*$^{-/-}$*;Sc5d*$^{-/-}$ and *Shh*$^{-/-}$*;7Dhcr*$^{-/-}$*;Sc5d*$^{-/-}$ clones, respectively, +/- s.e.m.

(E) The mosaic nEBs described in (C) were stained with either CMFDA Green (*Ptch1*$^{LacZ;LacZ}$*;Ptch2*$^{-/-}$*;Shh*$^{-/-}$) or CMAC Blue (*Shh*$^{-/-}$*, Shh*$^{-/-}$*;7Dhcr*$^{-/-}$*, Shh*$^{-/-}$*;Sc5d*$^{-/-}$*, and Shh*$^{-/-}$*;7Dhcr*$^{-/-}$*;Sc5d*$^{-/-}$) cell tracker dye prior to aggregation. Images were taken after 48 h of culture with a 20x objective. CMAC Blue dye was recolored in magenta. Genotypes are shown in rectangles in the respective color.

(F) Unesterified sterols were stained with filipin in nEBs of *Shh*$^{-/-}$ and *Shh*$^{-/-}$ cells with mutations in the *7Dhcr* and/or *Sc5d* gene after 1 (A-D), 2 (A'-D'), and 3 days (A''-D''). Images were taken with a 63x objective. **** $p < 0.0001$; n.s. = not significant


A
mock
7DHC
****
Ptch1:LacZ expression normalized to mock
1.2
1
.8
.6
.4
.2
100% Ptch1LacZ/LacZ;Ptch2-/-;Shh-/-
50% Ptch1LacZ/LacZ;Ptch2-/-;Shh-/-
50% Shh-/-
B
transfection
Ptch1-/-;Ptch2-/-
or
Ptch1-/-;Ptch2-/-;7Dhcr-/-
fibroblasts
medium transfer
Light2 cells
Conditioned medium from
Ptch1-/-;Ptch2-/- fibroblasts
Ptch1-/-;Ptch2-/-;7Dhcr-/- fibroblasts
Gli luciferase expression normalized to mock
*
mock
Ptch1
Ptch2


C
Hh response:
7Dhcr+/+
high
7Dhcr-/-
low
1:1 mix
exactly in the middle?
Null hypothesis (H0): the Hh response in mosaic organoids with 50% 7Dhcr-/- and 50% 7Dhcr+/+ cells will be the average of the individual organoids
Ptch1:LacZ expression normalized to mock
****
null hypothesis
Ptch1LacZ/LacZ;Ptch2-/-;Shh-/-

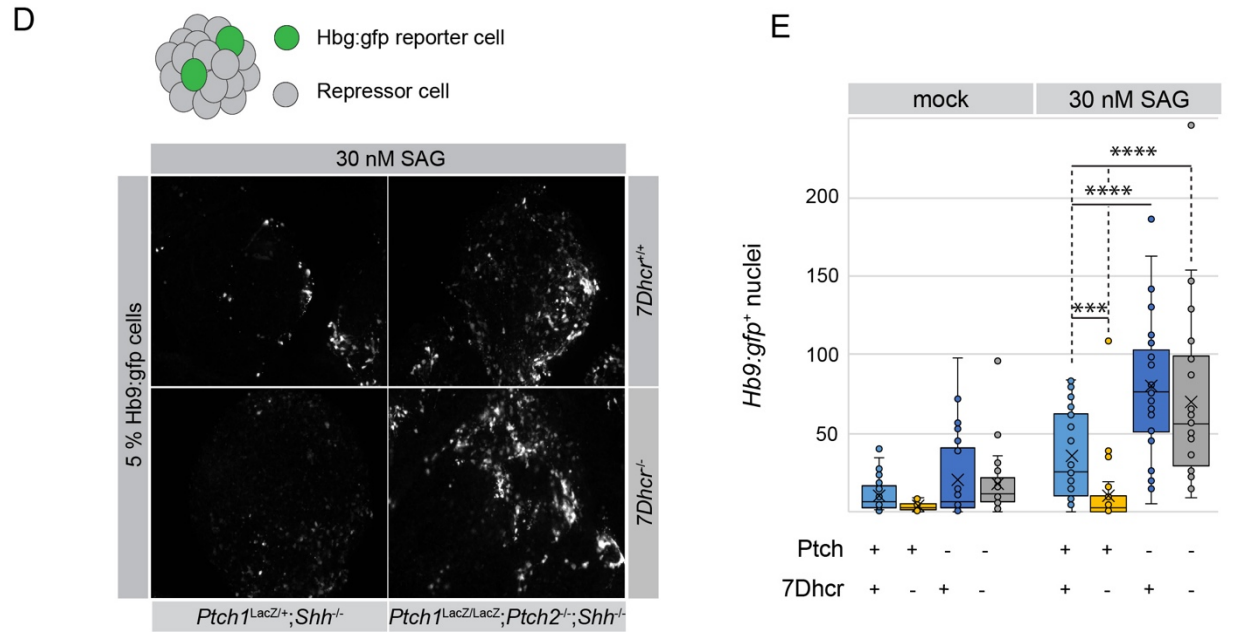

D
Hbg:gfp reporter cell
Repressor cell
30 nM SAG
5 % Hb9:gfp cells
7Dhcr+/+
7Dhcr-/-
Ptch1LacZ/+;Shh-/-
Ptch1LacZ/LacZ;Ptch2-/-;Shh-/-
E
mock
30 nM SAG
****
****
***
Hb9:gfp+ nuclei
200
150
100
50
Ptch + + - - + + - -
7Dhcr + - + - + - + -

Figure 3: Non-cell autonomous inhibition by late cholesterol precursors requires Ptch1/2.
A: *Ptch1:LacZ* measurement of *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$*;Shh*$^{-/-}$ reporter cells in SCOs or in mosaic SCOs with 50 % *Shh*$^{-/-}$ mESCs after 3 days of serum starvation. 13 µM 7DHC was added after the first night of SCO aggregation. Ptch1:LacZ levels are normalized to the respective mock treatment to account for lower numbers of reporter cells and inhibition by Ptch1/2 in mosaic SCOs. Box-and-Whisker plot, n=4. **B:** Experimental setup and Gli luciferase measurements of Hh responsive Light2 cells. *Ptch1*$^{-/-}$*;Ptch2*$^{-/-}$ or *Ptch1*$^{-/-}$*;Ptch2*$^{-/-}$*;7Dhcr*$^{-/-}$ fibroblasts were transfected with mock, *Ptch1*, or *Ptch2* and their conditioned medium was filtered and transferred onto Light2 cells with 30 nM SAG. Gli luciferase levels were normalized to Renilla luciferase and all conditions to their respective mock transfection. Box-and-Whisker plot, n=4. **C:** Graphic representation of the null hypothesis (indicated by red line): If Ptch1/2 is not required for non-cell autonomous inhibition, *Ptch1:LacZ* expression in mosaic SCOs will be the average of the individual SCOs. *Ptch1:LacZ* measurement of *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$*;Shh*$^{-/-}$*, Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$*;Shh*$^{-/-}$*7Dhcr*$^{-/-}$*,* and mosaic SCO consisting of equal amounts of the two reporter lines 3 days after serum starvation. Box-and-Whisker plot, n=4. **D:** Mosaic SCOs with 5% *Hb9:gfp* reporter cells and the indicated repressor cells were serum starved for 3 days and treated with 30 nM SAG for the last 24 hours. SCOs were fixed and *Hb9:gfp* expression imaged. Shown are representative images of mosaic SCOs for each condition. **E:** Quantification of *Hb9:gfp* positive nuclei of the experiment shown in D, shown in a Box-and-Whisker plot, n=3.* $p<0.05$, *** $p<0.001$, ****$p<0.0001$

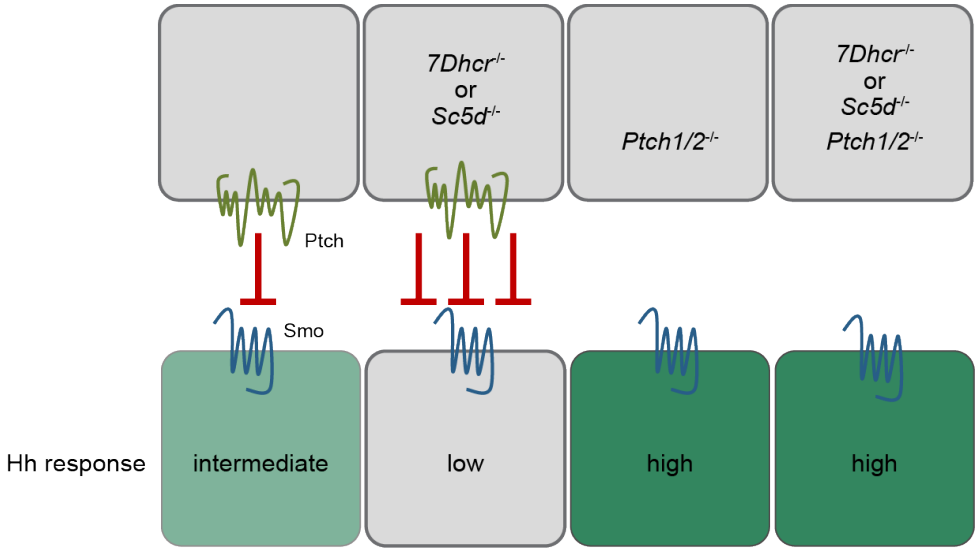


Figure 4: Model of non-cell autonomous Smo inhibition by Ptch1/2.
Ptch1/2 inhibits Smo non-cell autonomously and does so more potently when the Ptch1/2-containing cells lack enzymes of cholesterol synthesis, resulting in a decreased transcriptional Hh response. In the absence of Ptch1/2 and independent of the absence or presence of enzymes of cholesterol synthesis, Smo in uninhibited and the transcriptional Hh response is high.

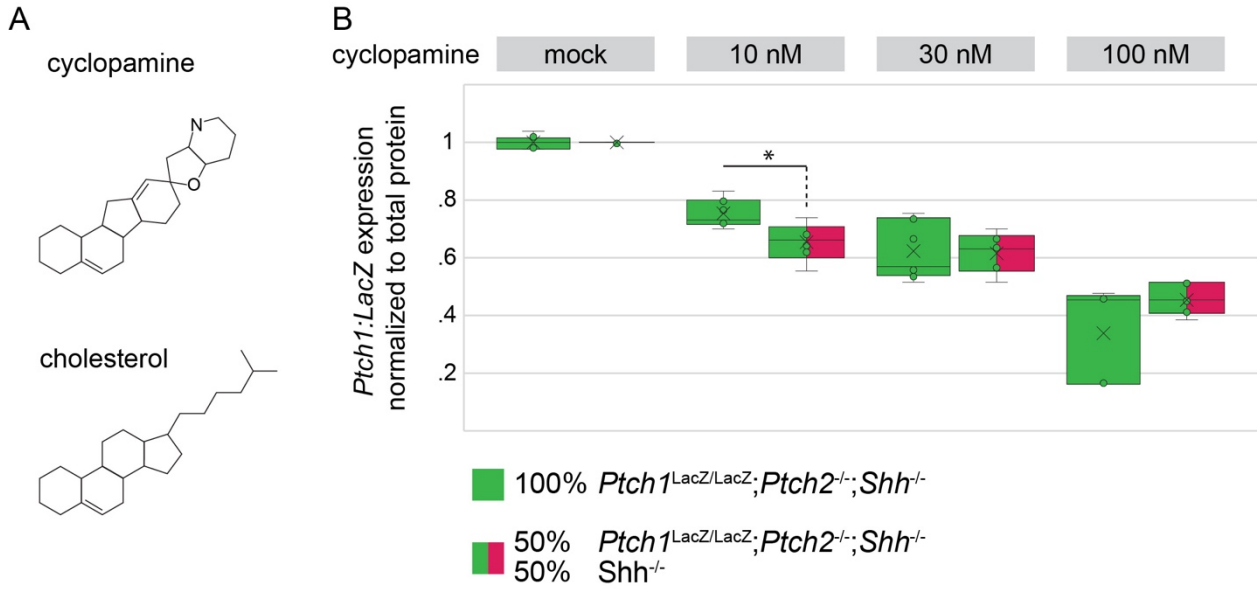


Figure S1: Ptch1/2 does not enhance the inhibition of Smo by cyclopamine.
A: Structural comparison of the steroidal molecules cyclopamine and cholesterol. **B:** *Ptch1:LacZ* measurement of *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$;*Shh*$^{-/-}$ reporter cells in SCOs or in mosaic SCOs with 50 % *Shh*$^{-/-}$ mESCs after 3 days of serum starvation. The indicated concentrations of cyclopamine were added after the first night of SCO aggregation. *Ptch1:LacZ* levels are normalized to the respective mock treatment to account for lower numbers of reporter cells and inhibition by Ptch1/2 in mosaic SCOs. Box-and-Whisker plot, n=6, *p<0.05.