JCB

Sterol and oxysterol synthases near the ciliary base activate the Hedgehog pathway

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Re: JCB manuscript #202002026

Dr. David Ronan Raleigh
University of California San Francisco
Radiation Oncology and Neurological Surgery
Helen Diller Family Cancer Research Building
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San Francisco, CA 94158

Dear Dr. Raleigh,

Thank you for submitting your manuscript entitled "Sterol and oxysterol synthases at the ciliary base activate the Hedgehog pathway" to the Journal of Cell Biology. The manuscript has now been assessed by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

Unfortunately, you will see that all three reviewers have major scientific concerns that cannot be easily addressed with a few key experiments. While all find the ciliary base localization studies convincing and interesting, none are convinced that Smo is required for the DHCR7 function based on the data presented.

Unfortunately I do not have the level of reviewer support that I would need to proceed further with the paper. I do realize that significant further work and expansion might convincingly address some of these issues, but I am hesitant to encourage you to work towards the aim of further consideration at JCB. The level of reviewer criticism makes it impossible for me to guarantee that we will be able to invite resubmission, even after revision. Therefore, it does seem that it will be best for you to consider another journal for this work. Our journal office will transfer your reviewer comments to another journal upon request.

I am sorry our decision is not more positive, but hope that you find the reviews constructive. Of course, this decision does not imply any lack of interest in your work and we look forward to future submissions from your lab.

Thank you for your interest in the Journal of Cell Biology.

Sincerely,

Maureen Barr, Ph.D.
Monitoring Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This manuscript by Findakly examined the cellular localization of the cholesterol generating enzyme DHCR7 and the oxysterol synthase CYP7A1 at the base of cilia during Hedgehog signaling. They find that DHCR7 is localized at the base of cilia and its activity and its level at the ciliary base is reduced upon Hedgehog pathway activation. In contrast, they find that Hedgehog pathway activation stimulates CYP7A1 and promotes its activity at the ciliary base. The localization studies are well done, nicely quantified and convincing. On the other hand, the effects of these enzymes on the regulation of the pathway are not clear and the model presented in Figure 5 F and G is not helpful in understanding the results. If DHCR7 makes cholesterol when the pathway is off, and cholesterol activates Smo why would this be active in the off state? Likewise, why would CYP7A1 be an activator if it converts 7-DHC to the inactive sterol 7k-C? Is CYP27A1 required to convert the 7k-C to an active sterol? In short, the manuscript needs significant rewriting and probably more experimentation to clarify the role of these enzymes in the pathway.

The loss of DHCR7 causes basal expression of Gli1 to drop to about 1/4 of normal (Figure 1E) but SHH induced expression drops about the same amount (Figure 1H) leaving overall induction to be about the same. This suggests that DHCR7 may have roles in regulating GliR levels. Have these been examined?

The idea that Smo is required for the DHCR7 function is not convincing. This is based on the weak data in Figure 1I showing that basal Gli1 expression goes up in Smo-/- cells when both DHCR7 and Smo is transfected in. Why would you expect Smo to play a role in this since Smo would not be expected in the cilium at the off state?

It seems remarkable to me that enzymes localized to such tight foci in the cell can have the dramatic effects on cholesterol and sterols as reported by the authors. For example, cholesterol levels drop by about 1/3 in Figure 1F. Are the images so highly contrasted that much of the signal of these enzymes is not visible in the figures?

The cycloheximide experiments are superficial and need further work to establish that they are actually having the expected effect. Some discussion of why you would expect post-transcriptional regulation would be helpful.

The Ofd1 experiments need controls showing that the phenotype can be rescued by re-expression of Ofd1. In text referring to this experiment, I think the authors mean centriole rather than centrosome, as I am not aware of centrosomes having defined lengths and distal structures.

Figure presentation needs improvement. Most composite figures include white for one of the merged colors. This is problematic because it obscures the red, which is usually the color that one cares about in the image.

The use of * to designate significance needs work. Figure 2J uses both * and ** to designate p=0.001. Some figure use more *'s to designate more significance while others use fewer *'s to designate more significance. In some panels, the number of *'s is random with regard to the level of significance.

Figure 3B appears to have incorrectly labeled CEP170 and AcTub colors.

Were gene edited clones or populations were used for the figures? The text in the results suggests uncloned populations but the materials and methods suggests clones. This is important for interpretation of population assays where Gli1 expression was measured by RT-PCR or cholesterol was measured.

Reviewer #2 (Comments to the Authors (Required)):

In their manuscript, "Sterol and oxysterol synthases at the ciliary base activate the Hedgehog pathway", Findakly et al., establish the presence of two regulators of the Hedgehog pathway at the base of the cilium. There's strong evidence that cholesterol and other oxysterols are capable of modulating and activating Smoothened. However, the link between these molecules and the cilium, and the rest of the Hedgehog pathway, is still being worked out.
Here, the authors report that cholesterol synthase DHCR7 and oxysterol synthase CYP7A1 localize with the base of the primary cilium and promote Hedgehog signaling. However, DHCR7 and CYP7A1 are differentially regulated by Hedgehog activation. Hedgehog pathway activation results in increased CYP7A1 and decreased DHCR7 at the ciliary base. Furthermore, CYP7A1 is required for Shh-induced Smoothened ciliary accumulation and activation of Shh-dependent genes. These data move the field forward as they provide a potential geographical link between cilia, Smoothened, and the endogenous production of sterols and oxysterols relevant to Smoothened activation.
Several issues need to be addressed by the authors:
1) The authors need to examine their statistical methods, reanalyze data in the manuscript and state what test is used for the distinct situations. (All comments below assume that the same data are significant upon reanalysis.) For example, the use of a student's t-test is not appropriate in all situations. Data sets with three or more groups should be analyzed by a one-way ANOVA. Examples of this are Figure 4A and B. When there are two variables within each group, a two-way ANOVA, would be appropriate. Examples of this include 4E, F and 5B-D. These examples are non-inclusive.
a. Moreover, p-values are not effect sizes and give no indication of the magnitude of a difference, so reporting them in this way is unnecessary. A consistent use of asterisk combinations across figures would be more straightforward and easier for the reader to follow.

2) The manuscript is missing critical experimental details. These details are needed both for straightforward reader comprehension and for experimental replication.
a. The CRISPRi should be explained a bit more and include a statement that the purpose of dCas9-KRAB is to repress expression. The methods section identifies the multiple guide RNAs for each gene of interest, but there's no clear indication of where they bind relative to one another or to transcription start sites so their relevance to the cell lines used in each experiment is murky. It's also unknown where the qPCR primers are located relative to the guide RNAs so it is not possible to determine whether the primers are appropriate. A diagram, even if supplemental, including this information for each locus would help.
b. In addition, the methods section lays out three separate pairs of sgDhcr7 guides and alludes to 3 cell lines, but at most two cell lines are analyzed. In some experiments, only a single sgDhcr7 cell line is chosen, but which one and why it was chosen is unclear. The same is true of sgCyp7a1 and sgPtch1 cell lines. What cell lines are used for what experiments? Why are some excluded?

3) The association of these synthases with the ciliary base is curious. Are there known substrates present at the base of the cilium that could be regulating DHCR7? How do the authors envision these enzymes working relevant to Hh signaling? As a Report, the full mechanism need not be in

place but the work needs to be of high general interest, which in this case requires some speculation based on the novel observations described.
4) The authors point out that their observations "perhaps explain why conflicting studies have define DHCR7 as either a positive or negative regulator of the Hedgehog pathway." While true, the authors observed changes occur after 24 hours of treatment with SAG or Shh, so it's possible that there are distinct temporal changes not detected here- or that fibroblasts may not reveal the full dynamic range of the Hedgehog pathway. Recent precedence for this is reflected in analysis of INPP5E function in fibroblasts verses embryos. The authors should discuss this possibility.

5) The proposed model is that DHCR7 presence at the ciliary base is negatively regulated by Hh pathway activation. How do the authors reconcile their observation that tagged DHCR7 accumulates at the base of cilia in Ptch1-¬/- cells where the Hh pathway is constitutively activated with this model?

6) In fact, the data presented could support an alternate model where DHCR7 and CYP7A1 control Smo entry into cilia, rather than Smo accumulation along the ciliary membrane. Sterols are known to alter Smo confirmation and therefore could be having an impact on Smo trafficking dynamics outside of accumulation in cilia.
a. Treatment of cells with sgCyp7a1 decreases Smo accumulation in cilia. DHCR7 appears to have a synergistic relationship with Smo to promote Shh-dependent gene transcription. How do manipulations of DHCR7 influence Smo localization to cilia? This is important to understand the authors' model of the feedback of Hh to DHCR7.

7) Gli1 transcript levels should all use the same units and the display of those data should be the same. The experiment in 5B is missing appropriate controls, which are present in 4F.

8) The figure legend for S1 appears to be incorrect. The text states that cells in S1B are Ptch-/-, but the figure legend identifies them as MEFs. The text could be clarified in alignment with the labels.

Reviewer #3 (Comments to the Authors (Required)):

In this manuscript, Findakly et al. present evidence that enzymes involved in sterol metabolism localize to the ciliary base and regulate Hedgehog signaling. This is a timely study, as understanding how cholesterol and oxysterols regulate Smoothened activity and Hedgehog (Hh) signaling is a key unanswered question. This paper focuses on a key aspect of this question: how the local lipid environment of the ciliary membrane is regulated to control the activity of sterol-binding proteins such as Smo.

The most compelling and novel aspect of this manuscript is the finding that sterol biosynthetic enzymes Dhcr7 and Cyp7a1 - both previously linked to Hh signaling - localize to the ciliary base in a manner that is regulated by Hh pathway activity. Combined with evidence that Hh signaling is decreased when these enzymes are knocked down, the authors propose that Hh signaling depends upon enzymes that locally tune the sterol composition of the ciliary membrane. Such a model would provide much-needed insight into how the ciliary environment regulates Smo activity and enables Hh signaling. However, in my opinion the current data do not provide sufficient support for this model. Two key limitations are 1) the genetic perturbations and lipidomic analyses are done at the whole-cell level and thus do not address whether Dhcr7 and Cyp7a1 have local effects on cilia or global effects on the whole cell, and 2) while these enzymes appear to localize to the 'ciliary base', where exactly they are found within this area is not established, leaving it unclear exactly

if/how they can alter the sterol composition of the ciliary membrane. While addressing these concerns may require significant additional data, doing so could yield a rigorous paper of broad interest to JCB's readership.

Major points

1. The model that dynamic relocalization of sterol enzymes modulates signaling through local changes in lipid levels is attractive but suffers from a critical limitation. While the CRISPRi data indicate that Dhcr7 and Cyp7a1 influence Hh signaling, they do not demonstrate that the pool of enzyme at the ciliary base is functionally relevant (indeed prior reports have already established that these enzymes influence Hh signaling, although notably Kinnebrew et al find Cyp7a1 as a negative rather than positive regulator of signaling). Similarly, the lipidomics data indicate that whole-cell sterol levels change upon enzyme knockdown (or when pathway activity is altered by Ptch1 disruption) but don't provide evidence that there are distinct local effects within the ciliary membrane. Addressing these points may benefit from probes for detecting ciliary vs plasma membrane cholesterol or for manipulating Dhcr7/Cyp7a1 localization at the ciliary base (although these tools may not be readily available or as robust as reagents used to establish local phosphoinositide regulation in cilia). Finally, it is surprising that loss of Dhcr7 from the ciliary base (e.g. upon pathway activation in Ptch1 KO cells or upon Ift88 KO) is sufficient to cause whole-cell levels of e.g. 7-DHC to change. Indeed Ptch1 CRISPRi leads to a 7-DHC increase of comparable magnitude to Dhcr7 knockdown (Fig. 1F vs 2H). Such an outcome might be expected if the enzyme pool at the ciliary base is the main/exclusive active pool of enzyme, or if Ptch1/Ift88 disruption alters Dhcr7 activity outside of the ciliary base, or if Ptch1/Ift88 also regulate other enzymes in sterol metabolism. With this variety of possible explanations, the functional significance of Dhcr7 removal from the ciliary base is unclear.

2. Dhcr7 and Cyp7a1 are both transmembrane enzymes, and thus their localization to the ciliary base must be in the context of a nearby membrane bilayer. However, the localization data presented here do not make it clear where exactly these enzymes are found (i.e. are they in the ciliary membrane, the transition zone membrane, the ciliary pocket, or vesicles near the basal body?). Resolving this issue would increase confidence that these enzymes are in fact present at the ciliary base and would clarify if/how their local activity might alter the sterol content of associated membranes (and the sterol pool acting on ciliary Smo). More specific issues are that the localization of Myc-Dhcr7 in Fig. S1B appears different from that of endogenous Dhcr7, and the localization of Cyp7a1 often looks unusual and possibly distinct from where membranes might be expected (e.g. proximal or adjacent to the Cep170 signal in Fig. 3). Lastly, can the authors clarify why Dhcr7 staining does not reveal pronounced ER and Golgi localization as previously reported?

Minor points

1. Introduction: Do Cyp7a1 and Hsb11b2 actually carry out the same reaction as stated? It appears the substrates are different, and the product of Cyp7a1 may be the 7-hydroxy rather than 7-keto form?

2. Fig 1F-H: Which exact sgRNA pair was used for these experiments? Can the authors quantify the results for Fig. 1G as in Fig. 2A (the other cells in Fig S1E don't appear to show as pronounced a decrease)?

3. Fig. 4A-B. What does it mean that Cyp7a1 knockdown reduced signaling in unstimulated cells? This is presumably a case where Cyp7a1 is not localized to the ciliary base.

4. Fig. 4C-F: Which exact sgRNA was used?

5. In some experiments, data are presented from one clonal CRISPRi knockdown. For select cases such as Fig. 4D-F, it would be more rigorous to show data from additional clones or sgRNAs.

6. Fig. S3A: Are the panel labels or coloring wrong? It would be helpful to note in the text that Ptch1 KO MEFs were used.

7. Can the authors include the data for cholesterol levels related to Fig. 2H-J and comment on why they may be changed or not?

8. Discussion: It would be worth noting that Cyp7a1 KO mice don't have an overt Hh phenotype (PMIDs 12588590 and 12093894).

1st Revision - Authors' Response to Reviewers: August 12, 2020

Thank you for the time you have spent evaluating our manuscript, **Sterol and oxysterol synthases at the ciliary base activate the Hedgehog pathway**. We were delighted to read that our studies are "timely," "move the field forward," provide a "geographical link between cilia, Smoothened, and the endogenous production of sterols and oxysterols relevant to Smoothened activation," and that our "localization studies are well done, nicely quantified and convincing." To address the reviewers' concerns, we have added new data and clarified our results in the context of published literature in this revision. The reviewer critiques, and our point-by-point responses in blue text, are delineated below.

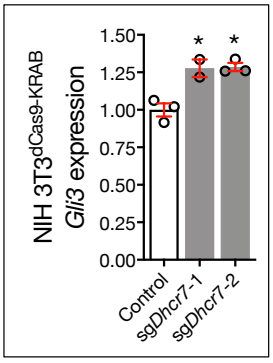
Reviewer 1

This manuscript by Findakly examined the cellular localization of the cholesterol generating enzyme DHCR7 and the oxysterol synthase CYP7A1 at the base of cilia during Hedgehog signaling. They find that DHCR7 is localized at the base of cilia and its activity and its level at the ciliary base is reduced upon Hedgehog pathway activation. In contrast, they find that Hedgehog pathway activation stimulates CYP7A1 and promotes its activity at the ciliary base. The localization studies are well done, nicely quantified and convincing. On the other hand, the effects of these enzymes on the regulation of the pathway are not clear and the model presented in Figure 5 F and G is not helpful in understanding the results. If DHCR7 makes cholesterol when the pathway is off, and cholesterol activates Smo why would this be active in the off state? Likewise, why would CYP7A1 be an activator if it converts 7-DHC to the inactive sterol 7k-C? Is CYP27A1 required to convert the 7k-C to an active sterol? In short, the manuscript needs significant rewriting and probably more experimentation to clarify the role of these enzymes in the pathway.

We are grateful for the support of our studies, and have significantly rewritten our manuscript for this revision, adding new experimentation and clarifying our results in the context of published literature to address these and the following critiques. In our concluding paragraphs, we have clarified our results showing that DHCR7 is a positive regulator of the Hedgehog pathway (also supported by PMID 12914579) that is inhibited by pathway activation. These results suggest that DHCR7 functions reciprocally to PTCH1, which is a negative regulator of the Hedgehog pathway that is nevertheless a positive target of the Hedgehog transcriptional program. Although DHCR7 indeed produces more cholesterol when the pathway is off (Figure 1 F), endogenous Smoothened is not present in the primary cilium in the absence of Hedgehog ligands (PMID 16136078). In support of this mechanism, we have performed new experiments for this revision showing that Smoothened accumulation in the primary cilium in response to Hedgehog ligands does not require DHCR7 (Figure 1 K). Similarly, we now show that Smoothened accumulation in the primary cilium in response to Hedgehog ligands does not require CYP7A1 (Figure 4 D). Conversely, Smoothened is required for DHCR7 to activate the Hedgehog transcriptional program (Figure 1 J), and we have performed new experiments for this revision showing that over-expression of either DHCR7 or CYP7A1 activate the Hedgehog transcriptional program (Figure S3 E). Thus, as is now reflected in the revised model in Figure 5 G, these data are consistent with DHCR7 priming the lipid microenvironment of the primary cilium for Smoothened activation, which occurs once Smoothened accumulates in the primary cilium in response to Hedgehog ligand stimulation. Consistent with this model, we have previously shown that primary cilia are enriched in cholesterol even in the absence of Hedgehog pathway activation (PMID 39349923, Figure S1 C), an important finding that we now reference in the concluding paragraphs of our revised manuscript. Further, we have also previously shown that CYP27A1 is required to convert 7k-C to an active oxysterol, and is expressed in MEFs, the developing cerebellum, and other cells and tissues that transduce ciliary Hedgehog signals (see Figure 4 in PMID 39349923). Thus, we have clarified the important function of CYP27A1 in the legend of Figure 5. In summary, and as described in further detail below, we have significantly rewritten our manuscript, revised existing figures, and added new experimentation to this revision that refine our understanding of DHCR7 and CYP7A1 cellular functions in the context of Hedgehog signal transduction, all of which support our conclusions that both enzymes promote activation of the Hedgehog pathway.

The loss of DHCR7 causes basal expression of Gli1 to drop to about 1/4 of normal (Figure 1E) but SHH induced expression drops about the same amount (Figure 1H) leaving overall induction to be about the same. This suggests that DHCR7 may have roles in regulating GliR levels. Have these been examined?

We have generated new data for this revision (presented to the right), showing that expression of *Gli3* (which has a repressive effect on the Hedgehog transcriptional program) only increases nu 25% after loss of *Dhcr7*. Although statistically significant, these changes are unlikely biologically significant in the context of the more dramatic



decreases in expression of *Gli1* (which has an activating effect on the Hedgehog transcriptional program) after loss of *Dhcr7* (Figure 1 E). Moreover, GLI3 is not known to have a direct impact on Smoothened function, and we now show that Smoothened is necessary for DHCR7 to activate the Hedgehog transcriptional program (Figure 1 J). These data suggest that GLI repressor functions do not significantly contribute to the impact of DHCR7 on Hedgehog signal transduction.

The idea that Smo is required for the DHCR7 function is not convincing. This is based on the weak data in Figure 1I showing that basal Gli1 expression goes up in Smo-/- cells when both DHCR7 and Smo is transfected in. Why would you expect Smo to play a role in this since Smo would not be expected in the cilium at the off state?

A limitation of Smoothened over-expression experiments is that exogenous Smoothened accumulates in the primary cilium even in the absence of pathway stimulation (PMID 39349923, Figure 2 H). However, for the Smoothened over-expression experiment shown in Figure 1 J of this revision (formerly Figure 1 I), unstimulated Smoothened localization to the primary cilium in *Smo*$^{-/-}$ MEFs was of benefit insofar as it allowed us to epistatically map the functional relationship between DHCR7 expression and activation of the Hedgehog transcriptional program to Smoothened. We have clarified this important technical detail in our revised Results. Moreover, as mentioned above (reviewer 1, response 1), we have now added new quantitative immunofluorescence data using multiple sg*Dhcr7* monoclonal NIH 3T3 CRISPRi cell lines demonstrating that suppression of *Dhcr7* does not block Smoothened from accumulating in the cilium in response to Hedgehog ligands (Figure 1 K). Consistently, using sg*Cyp7a1* monoclonal NIH 3T3 CRISPRi cell lines, we now show that suppression of *Cyp7a1* does not block Smoothened from accumulating in the cilium in response to Hedgehog ligands (Figure 4 D). These data further refine our understanding of the functional impact of DHCR7 and CYP7A1 activity on the Hedgehog pathway, and demonstrate that removal of DHCR7 from the ciliary base does not merely allow Smoothened to enter the cilium. Rather, the new data we have generated for this revision decouple Smoothened localization from Smoothened activation, the latter of which is regulated by DHCR7 activity. We have added our interpretation of data to the Results.

It seems remarkable to me that enzymes localized to such tight foci in the cell can have the dramatic effects on cholesterol and sterols as reported by the authors. For example, cholesterol levels drop by about 1/3 in Figure 1F. Are the images so highly contrasted that much of the signal of these enzymes is not visible in the figures?

Our images are not so highly contrasted as signal from these enzymes are not visible elsewhere in cells. For instance, in the first row of Figure S1 A, endogenous DHCR7 can be seen in a peri-nuclear/ER pattern, consistent with the previously reported localization pattern of DHCR7 (PMID 30535733) that we now recapitulate with new whole cell DHCR7 (and CYP7A1) over-expression immunofluorescence experimentation (Figures S1 D and S3 B). We also generated new data for this revision showing that over-expression of DHCR7 and CYP7A1 induce the Hedgehog transcriptional program (Figure S3 E). Interestingly, we find different patterns of expression for endogenous and over-expressed DHCR7 and CYP7A1 proteins that appear to be influenced by transformation technique or antibody/tag pairs (Myc vs HA). These data underscore the importance of interrogating endogenous gene products when studying the Hedgehog pathway (as we have done throughout out manuscript), and perhaps shed light on the often-contradictory literature relating to the impact of DHCR7 on Hedgehog signal transduction (PMID 26685159, 16687448, 31657721). Finally, as the rate limiting step in cholesterol biosynthesis, it is unsurprising that cholesterol levels would dramatically change upon suppression of DHCR7 (PMID 12543708). We have clarified the important function of DHCR7 for cholesterol biosynthesis in the Introduction.

The cycloheximide experiments are superficial and need further work to establish that they are actually having the expected effect. Some discussion of why you would expect post-transcriptional regulation would be helpful.

In the Results, we have clarified that the Hedgehog pathway regulates development through (i) gene expression programs, and also (ii) signaling events that are independent of gene expression (PMID 23719536). Further, we showed that Hedgehog pathway activation does not change the expression of DHCR7 (Figure S2) or CYP7A1 (Figure S3C), suggesting that expression of intermediate regulatory proteins functioning between Hedgehog pathway activation and DHCR7/CYP7A1 localization/activation are important for the biochemical mechanism our studies reveal. Indeed, we shave previously shown that some functions of the Hedgehog pathways (such as cell cycle progression) involve expression of indirect target genes requiring translation of intermediate transcription factors (see Figure 2 A in PMID 29202464). Thus, we used cycloheximide to shed light on the biochemical impact of Hedgehog signaling on DHCR7 and CYP7A1 localization, and we have clarified the

rationale for this experiment in the revised Results. In the Methods, we have also clarified that we used a dose and duration of cycloheximide that we have previously established as effective for blocking the expression of indirect targets of the Hedgehog transcriptional program in NIH 3T3 cells (see Figure 2 A in PMID 29202464). Consistently, we performed immunofluorescence for DHCR7 and CYP7A1 with cycloheximide ± SAG in the same experiment, and saw an impact on DHCR7 localization, but not on CYP7A1 localization. Thus, these experiments were (i) internally controlled (and we have unified these data in our revision in Figure 5 C to make this clearer); (ii) consistent with previously published and analogous experiments in terms of technical considerations, and (iii) motivated by fundamental mechanisms of Hedgehog signal transduction.

The Ofd1 experiments need controls showing that the phenotype can be rescued by re-expression of Ofd1. In text referring to this experiment, I think the authors mean centriole rather than centrosome, as I am not aware of centrosomes having defined lengths and distal structures.

We have corrected all instances of "centrosomes" in our initial submission to "centriole" in this revision, and thank the reviewer for bringing this mistake to our attention. Moreover, we have replaced the suboptimal confocal microscopy images in Figure 5 D from our initial submission with improved images of DHCR7 localization in wild type and *Odf1*^{Gt} mouse embryonic stem cells. Transfection of mouse embryonic stem cells is inefficient, and as we show with new experimentation in this revision, over-expression of exogenous proteins does not recapitulate the endogenous localization of DHCR7 or CYP7A1 (Figures S1 D and S3 B). Given these technical concerns, it is unlikely that re-expression of *Ofd1* (even if technically feasible) would yield interpretable results, or would inform our understanding of sterol synthase localization at centrioles. However, as mentioned above, we now provide improve confocal microscopy demonstrating that DHCR7 appropriately localizes to the centriole in control wild type mouse embryonic stem cells (Figure 5 D), which express OFD1.

Figure presentation needs improvement. Most composite figures include white for one of the merged colors. This is problematic because it obscures the red, which is usually the color that one cares about in the image.

We have revised the immunofluorescence presentation throughout our figures to show only red and green at the ciliary base, reserving white for the primary cilium itself, where appropriate. We thank the reviewer for this insightful suggestion.

The use of * to designate significance needs work. Figure 2J uses both * and ** to designate p=0.001. Some figure use more *'s to designate more significance while others use fewer *'s to designate more significance. In some panels, the number of *'s is random with regard to the level of significance.

We have standardized our designations of statistical significance, with * denoting $p \leq 0.05$, ** denoting $p \leq 0.01$ and *** denoting $p \leq 0.001$. We have clearly defined these thresholds in the Figure Legends and Methods.

Figure 3B appears to have incorrectly labeled CEP170 and AcTub colors.

We have corrected this error, and thank the reviewer for bringing it to our attention.

Were gene edited clones or populations were used for the figures? The text in the results suggests uncloned populations but the materials and methods suggests clones. This is important for interpretation of population assays where Gli1 expression was measured by RT-PCR or cholesterol was measured.

Monoclonal gene edited cell lines were used for all experiments. We have clarified this in the Results and Methods.

Reviewer 2

In their manuscript, "Sterol and oxysterol synthases at the ciliary base activate the Hedgehog pathway", Findakly et al., establish the presence of two regulators of the Hedgehog pathway at the base of the cilium. There's strong evidence that cholesterol and other oxysterols are capable of modulating and activating Smoothened. However, the link between these molecules and the cilium, and the rest of the Hedgehog pathway, is still being worked out. Here, the authors report that cholesterol synthase DHCR7 and oxysterol synthase CYP7A1 localize with the base of the primary cilium and promote Hedgehog signaling. However, DHCR7 and CYP7A1 are differentially regulated by Hedgehog activation. Hedgehog pathway activation results in increased CYP7A1 and decreased DHCR7 at the ciliary base. Furthermore, CYP7A1 is required for Shh-induced Smoothened ciliary accumulation and activation of Shh-dependent genes. These data move the field forward as they provide a potential geographical

link between cilia, Smoothened, and the endogenous production of sterols and oxysterols relevant to Smoothened activation.

Thank you for the positive assessment of our work.

The authors need to examine their statistical methods, reanalyze data in the manuscript and state what test is used for the distinct situations. (All comments below assume that the same data are significant upon reanalysis.) For example, the use of a student's t-test is not appropriate in all situations. Data sets with three or more groups should be analyzed by a one-way ANOVA. Examples of this are Figure 4A and B. When there are two variables within each group, a two-way ANOVA, would be appropriate. Examples of this include 4E, F and 5B-D. These examples are non-inclusive. Moreover, p-values are not effect sizes and give no indication of the magnitude of a difference, so reporting them in this way is unnecessary. A consistent use of asterisk combinations across figures would be more straightforward and easier for the reader to follow.

We have now incorporated one- and two-way ANOVA tests into our analyses (were appropriate, as kindly suggested by the reviewer), and denoted these tests in relevant Figure Legends and Methods. Moreover, we have standardized our designations of statistical significance throughout our manuscript, with * denoting $p \leq 0.05$, ** denoting $p \leq 0.01$ and *** denoting $p \leq 0.001$. We have clearly defined these thresholds in the Figure Legends and Methods.

The manuscript is missing critical experimental details. These details are needed both for straightforward reader comprehension and for experimental replication. The CRISPRi should be explained a bit more and include a statement that the purpose of dCas9-KRAB is to repress expression. The methods section identifies the multiple guide RNAs for each gene of interest, but there's no clear indication of where they bind relative to one another or to transcription start sites so their relevance to the cell lines used in each experiment is murky. It's also unknown where the qPCR primers are located relative to the guide RNAs so it is not possible to determine whether the primers are appropriate. A diagram, even if supplemental, including this information for each locus would help. In addition, the methods section lays out three separate pairs of sgDhcr7 guides and alludes to 3 cell lines, but at most two cell lines are analyzed. In some experiments, only a single sgDhcr7 cell line is chosen, but which one and why it was chosen is unclear. The same is true of sgCyp7a1 and sgPtch1 cell lines. What cell lines are used for what experiments? Why are some excluded?

We have added a description of the mechanism and purpose of CRISPRi to the Results and Methods, and have clarified the binding site of each sgRNA relative to the TSS of each target gene in the Methods, which were significantly modified for this revision. In the Methods, we have also clarified that all qRT-PCR primers we used spanned exon/exon boundaries, and have provided the genomic coordinates for the binding site of each qRT-PCR primer pair.

With respect to the gene edited cell lines used in our study, we have revised the Figures, Figure Legends, and Methods to clearly denote which sgRNA sequences/cells were used for each experiment. Due to the highly reproducible experimental consistency of these unique reagents, not all cell lines were used for every downstream experiment after validating gene suppression and the impact of gene suppression on gene product localization and Hedgehog signal transduction. For some sterolomic experiments, pooled results from multiple monoclonal cell lines are presented, which is also clarified in the revised Methods.

The association of these synthases with the ciliary base is curious. Are there known substrates present at the base of the cilium that could be regulating DHCR7? How do the authors envision these enzymes working relevant to Hh signaling? As a Report, the full mechanism need not be in place but the work needs to be of high general interest, which in this case requires some speculation based on the novel observations described.

Thank you for inviting the opportunity to elaborating on these fascinating issues. We have expanded the concluding paragraph to include a discussion of the technical factors currently limiting interrogation of the subcellular localization of Smoothened-activating oxysterols. The available data indeed suggest that substrates for DHCR7 and CYP7A1 (i.e. 7-DHC) are present at the ciliary base, but probes for detecting cellular sterols and oxysterols are not readily available, and existing reagents are not as robust as those established for interrogating other lipids, such as phosphoinositides. Indeed, existing sterol probes may be confounded by protein interactions, appear to require tremendous cholesterol concentrations for binding, and may not bind to the plasma membrane in a cholesterol-dependent manner (PMID 30422112). We have incorporated a discussion of these limitations in the revised Discussion, stating "… significant technical advances are necessary to improve our understanding of

Hedgehog signaling and ciliary lipids. Chief among these is likely the improvement of cellular probes for localizing cellular cholesterol, which are not as refined as reagents to study phosphoinositides in primary cilia."

With respect to how DHCR7 and CYP7A1 function with respect to the Hedgehog pathway, we have revised the model and associated text at the end of our study (Figure 5 G), showing that our data are consistent with DHCR7 priming the cilium with cholesterol that activates Smoothened upon ciliary entry, and that the intensity of Smoothened activation may be refined by exchanging DHCR7 for CYP7A1, and cholesterol for oxysterols. We thank the reviewer for the opportunity to consider the implications of our findings in the larger mechanism of Hedgehog signal transduction.

The authors point out that their observations "perhaps explain why conflicting studies have define DHCR7 as either a positive or negative regulator of the Hedgehog pathway." While true, the authors observed changes occur after 24 hours of treatment with SAG or Shh, so it's possible that there are distinct temporal changes not detected here- or that fibroblasts may not reveal the full dynamic range of the Hedgehog pathway. Recent precedence for this is reflected in analysis of INPP5E function in fibroblasts verses embryos. The authors should discuss this possibility.

Thank you for this suggestion. We have incorporated these important caveats into the revised Discussion.

The proposed model is that DHCR7 presence at the ciliary base is negatively regulated by Hh pathway activation. How do the authors reconcile their observation that tagged DHCR7 accumulates at the base of cilia in Ptch1-/- cells where the Hh pathway is constitutively activated with this model?

We apologize for this error in our initial submission, and have revised the Results to reflect the fact that $DHCR7^{MYC}$ was studied in wild type MEFs, and not *Ptch1*$^{-/-}$ MEFs, as was originally (and incorrectly) stated in the text associated with Figure S1 B.

In fact, the data presented could support an alternate model where DHCR7 and CYP7A1 control Smo entry into cilia, rather than Smo accumulation along the ciliary membrane. Sterols are known to alter Smo confirmation and therefore could be having an impact on Smo trafficking dynamics outside of accumulation in cilia.

Thank you for this insightful alternative interpretation of our data. In response, we have generated new data for this revision showing that suppression of DHCR7 does not enhance Smoothened accumulation in primary cilia upon Hedgehog pathway stimulation (Figure 1 K). Consistently, we also now show that Smoothened accumulation in the primary cilium in response to Hedgehog ligands does not require CYP7A1 (Figure 4 D). Thus, our data are most consistent with DHCR7 and CYP7A1 regulating Smoothened activation, rather than localization.

Treatment of cells with sgCyp7a1 decreases Smo accumulation in cilia. DHCR7 appears to have a synergistic relationship with Smo to promote Shh-dependent gene transcription. How do manipulations of DHCR7 influence Smo localization to cilia? This is important to understand the authors' model of the feedback of Hh to DHCR7.

As described above, we provide new data showing that suppression of DHCR7 neither blocks nor enhances Smoothened accumulation in primary cilia upon Hedgehog pathway stimulation (Figure 1 K), and have elaborated on these findings in the revised Results.

Gli1 transcript levels should all use the same units and the display of those data should be the same. The experiment in 5B is missing appropriate controls, which are present in 4F.

We have clarified in the Methods that *Gli1* transcript levels are displayed with the same units after normalization to control conditions using the ΔΔCt method, and have verified that all qRT-PCR experiments are displayed in the same style. However, due to differences in absolute *Gli1* induction levels upon Hedgehog pathway stimulation in different cell lines (a well-known characteristic among Hedgehog responsive cells, perhaps most evident in this study between *Smo*$^{-/-}$ MEFs in Figure 1 J and NIH 3T3 cells in Figure 1 I) we are unable to standardize the scale of the y-axis for all qRT-PCR experiments. For this revision, we have also now provided new data addressing the requested controls in Figure 5 B.

The figure legend for S1 appears to be incorrect. The text states that cells in S1B are Ptch-/-, but the figure legend identifies them as MEFs. The text could be clarified in alignment with the labels.

Thank you for bringing this error to our attention, which we have corrected in the Results to indicate that the experiment in Figure 1 B was performed in MEFs.

Reviewer 3

In this manuscript, Findakly et al. present evidence that enzymes involved in sterol metabolism localize to the ciliary base and regulate Hedgehog signaling. This is a timely study, as understanding how cholesterol and oxysterols regulate Smoothened activity and Hedgehog (Hh) signaling is a key unanswered question. This paper focuses on a key aspect of this question: how the local lipid environment of the ciliary membrane is regulated to control the activity of sterol-binding proteins such as Smo.

We are grateful for the positive assessment of our studies.

The most compelling and novel aspect of this manuscript is the finding that sterol biosynthetic enzymes Dhcr7 and Cyp7a1 - both previously linked to Hh signaling - localize to the ciliary base in a manner that is regulated by Hh pathway activity. Combined with evidence that Hh signaling is decreased when these enzymes are knocked down, the authors propose that Hh signaling depends upon enzymes that locally tune the sterol composition of the ciliary membrane. Such a model would provide much-needed insight into how the ciliary environment regulates Smo activity and enables Hh signaling. However, in my opinion the current data do not provide sufficient support for this model. Two key limitations are 1) the genetic perturbations and lipidomic analyses are done at the whole-cell level and thus do not address whether Dhcr7 and Cyp7a1 have local effects on cilia or global effects on the whole cell, and 2) while these enzymes appear to localize to the 'ciliary base', where exactly they are found within this area is not established, leaving it unclear exactly if/how they can alter the sterol composition of the ciliary membrane. While addressing these concerns may require significant additional data, doing so could yield a rigorous paper of broad interest to JCB's readership.

Thank you for the positive assessment of the novelty and impact of our studies, and also for the insightful comments about our findings in the context of ciliary structure. We have previously performed mass spectrometry-based sterolomics on evolutionarily diverse primary cilia and cells (see Figures 1A-C, 4C-E, and S1 in PMID 39349923), and have identified cilia-associated Smoothened-activating oxysterols (including cholesterol and 7k-C) that are enriched in whole cell sterolomics from domains of high-level Hedgehog signaling in vertebrates (see Figures 5E and S4 in PMID 39349923). Oxysterols such as 7k-C, 7k,27-OHC and 7b,27-DHC downstream of CYP7A1 activity are amphipathic and can diffuse throughout cells (PMID 12543708), and our previous work indicates that whole cell sterolomics are a reliable surrogate for cilia-associated Smoothened-activating oxysterol levels (PMID 39349923). Unfortunately, the genetic perturbations necessary to recapitulate the experiments from our current study in model systems allowing for primary cilia isolation in biochemical quantities suitable for sterolomics (such as *Stongylocentrotus purpuratus*, *Salpingoeca rosetta, Nematostella vectensis, Chlamydomonas reinhardtii,* or *Sus scrofa* cells) are not currently possible. Moreover, it is unclear if the genomes of these organisms encode *Dhcr7* or *Cyp7a1* orthologs. We have clarified these technologic limitations in the concluding paragraph of our manuscript to contextualize our studies and, hopefully, inspire new technologic innovations for future interrogations of subcellular lipid distributions. Further, with these technical limitations in mind, we have moderated our hypotheses and conclusions to focus on lipid changes in the “ciliary microenvironment” (rather than the primary cilium itself).

With respect to whether or not DHCR7 truly regulates the lipid composition of ciliary membranes, in light of the aforementioned technical limitations we have generated new data for this revision showing that Smoothened accumulation in primary cilia is independent of DHCR7 (Figure 1 K). Consistently, we now show that Smoothened accumulation in the primary cilium does not require CYP7A1 (Figure 4 D). In conjunction with our results showing that Smoothened is required for DHCR7 to activate the Hedgehog transcriptional program (Figure 1 J); biochemical, molecular biology, and cell biology experiments demonstrating that the products of DHCR7 and CYP7A1 directly bind to Smoothened and activate the Hedgehog transcriptional program (PMID 30192502 and 39349923); and the longstanding observation that Hedgehog ligand-dependent translocation to cilia is essential for Smoothened activity (PMID 16136078), our findings support a model of DHCR7- and CYP7A1-based activation of Smoothened in the primary cilium, which we have revised in Figure 5 G.

With respect to exactly where within the microenvironment of the ciliary base these enzymes localize, we have generated new data for this revision using the IN/OUT assay (PMID 27493724) to determine if DHCR7, a transmembrane protein (as described below) localizes to the ciliary pocket. These new data (Figure 5 E) corroborate our confocal and super-resolution microscopy showing that DHCR7 is tightly restricted to the ciliary base, and further suggest that DHCR7 is indeed located within the ciliary pocket. Technologies to identify and understand biophysical mechanisms of sterol transfer from the ciliary base or pocket to the ciliary body do not exist, but as mentioned above, it is notable that the lipids produced by DHCR7 and CYP7A1 are amphipathic and

can diffuse throughout cells (PMID 12543708). Thus, as is now reflected in the revised Discussion and Figure 5 G, our data are consistent with a model where DHCR7 produces cholesterol from the ciliary pocket in the absence of Hedgehog signals to prime the ciliary microenvironment for Smoothened activation, and upon Hedgehog stimulation, CYP7A1 contributes to cilia microenvironment by generating Smoothened-activating oxysterols from the ciliary base.

The model that dynamic relocalization of sterol enzymes modulates signaling through local changes in lipid levels is attractive but suffers from a critical limitation. While the CRISPRi data indicate that Dhcr7 and Cyp7a1 influence Hh signaling, they do not demonstrate that the pool of enzyme at the ciliary base is functionally relevant (indeed prior reports have already established that these enzymes influence Hh signaling, although notably Kinnebrew et al find Cyp7a1 as a negative rather than positive regulator of signaling). Similarly, the lipidomics data indicate that whole-cell sterol levels change upon enzyme knockdown (or when pathway activity is altered by Ptch1 disruption) but don't provide evidence that there are distinct local effects within the ciliary membrane. Addressing these points may benefit from probes for detecting ciliary vs plasma membrane cholesterol or for manipulating Dhcr7/Cyp7a1 localization at the ciliary base (although these tools may not be readily available or as robust as reagents used to establish local phosphoinositide regulation in cilia). Finally, it is surprising that loss of Dhcr7 from the ciliary base (e.g. upon pathway activation in Ptch1 KO cells or upon Ift88 KO) is sufficient to cause whole-cell levels of e.g. 7-DHC to change. Indeed Ptch1 CRISPRi leads to a 7-DHC increase of comparable magnitude to Dhcr7 knockdown (Fig. 1F vs 2H). Such an outcome might be expected if the enzyme pool at the ciliary base is the main/exclusive active pool of enzyme, or if Ptch1/Ift88 disruption alters Dhcr7 activity outside of the ciliary base, or if Ptch1/Ift88 also regulate other enzymes in sterol metabolism. With this variety of possible explanations, the functional significance of Dhcr7 removal from the ciliary base is unclear.

As the reviewer intimates, probes for detecting cellular sterols are not readily available, and are not as robust as those established for interrogating ciliary phosphoinositides. Indeed, existing sterol probes, such as were used in Kinnebrew et al., may be confounded by protein interactions, appear to require tremendous cholesterol concentrations for binding, and may not bind to the plasma membrane in a cholesterol-dependent manner (PMID 30422112). We have incorporated these technologic limitations in the concluding paragraph.

As the rate limiting step in cholesterol biosynthesis (PMID 12543708), it is expected that cellular cholesterol levels would dramatically decrease upon suppression of DHCR7, leading to an associated buildup in DHCR7 substrates such as 7-DHC. We have clarified the important function of DHCR7 for cholesterol biosynthesis in the Introduction, and have also clarified that DHCR7 indeed localizes elsewhere besides the ciliary base in the Results (which is supported by Figure S1 A from our initial submission, new over-expression data generated for this revision that is shown in Figure S1 D, and PMID 30535733). We showed that neither genetic nor pharmacologic activation of the Hedgehog pathway alters DHCR7 transcript or protein levels (Figure S2 A-G), and to our knowledge, there are no data in the large body of literature exploring PTCH1 and IFT88 that hints these proteins regulate DHCR7 activity, either at or away from the ciliary base. Nevertheless, we cannot exclude the possibility that genetic perturbations targeting ciliary structure or critical regulators of the Hedgehog pathway do not influence the expression or activity of other enzymes. Indeed, we have previously shown that HSD11B2 is induced by oncogenic Hedgehog signaling (see Figure 3A in PMID 39349923), and we provide new data generated for this revision that suggest genetic deletion of *Ptch1* or *Ift88* (Figure S2 H, see second to last response below) activates the cholesterol shunt pathway (PMID 12543708). With these important caveats to our results in mind, we have revised the Results and concluding paragraphs to include a discussion of these considerations. In the latter, we acknowledge that “it remains to be established why DHCR7 removal from the ciliary base is a necessary component of this process, but it is notable that non-enzymatic oxidation products of 7-DHC, such as DHCEO, antagonize Smoothened (PMID 27162362). Thus, it is possible that accumulation of DHCEO at the ciliary base in the absence of DHCR7 opposes the activity of CYP7A1 and oxysterols on Smoothened, and functions to constrain the intensity of Hedgehog signaling after the pathway is activated. It also remains to be fully established to what extent genetic or pharmacologic perturbations targeting particular aspects of ciliary structure, Hedgehog signaling, or Smoothened-activating lipid biosynthesis reciprocally impact other facets of subcellular structure or enzyme activity. Indeed, we find that DHCR7 and CYP7A1 cooperative to activate the Hedgehog pathway, and that genetic perturbation of Hedgehog signaling and primary cilia may influence the activity of enzymes participating in the cholesterol shunt pathway. In sum, current paradigms of Hedgehog signaling and lipid biosynthesis are reminiscent of negative feedback loops that are well-established elsewhere in the Hedgehog pathway, such as induction of the pathway inhibitor *Ptch1* in response to activation of the Hedgehog

transcriptional program." In summary, in the context of the control experiments we present, and extensive primary literature, we respectfully disagree that our results could be explained by alternative models inspired only by the extent of enzymatic biproduct changes, which ignore the orthogonal experiments we present that corroborate the model presented in Figure 5 G.

Dhcr7 and Cyp7a1 are both transmembrane enzymes, and thus their localization to the ciliary base must be in the context of a nearby membrane bilayer. However, the localization data presented here do not make it clear where exactly these enzymes are found (i.e. are they in the ciliary membrane, the transition zone membrane, the ciliary pocket, or vesicles near the basal body?). Resolving this issue would increase confidence that these enzymes are in fact present at the ciliary base and would clarify if/how their local activity might alter the sterol content of associated membranes (and the sterol pool acting on ciliary Smo). More specific issues are that the localization of Myc-Dhcr7 in Fig. S1B appears different from that of endogenous Dhcr7, and the localization of Cyp7a1 often looks unusual and possibly distinct from where membranes might be expected (e.g. proximal or adjacent to the Cep170 signal in Fig. 3). Lastly, can the authors clarify why Dhcr7 staining does not reveal pronounced ER and Golgi localization as previously reported?

Please see our response regarding DHCR7 function and localization above (reviewer 3, response 2), specifically with respect to our new data generated for this revision with the IN/OUT assaying (Figure 5 E) showing that DHCR7 localizes to the ciliary pocket. We agree with the reviewer that DHCR7 is a 9-pass transmembrane protein (PMID 12914579), and may localize to vesicles at the ciliary base. In contrast, the assertion that CYP7A1 is a transmembrane protein is speculative and based on the potential homology of a short, 24-residue region at the N-terminus of the protein, which has not been validated and could be the target of proteolytic degradation (PMID 24927729). Consistent with the likelihood that CYP7A1 is not anchored to the membrane, the localization of CYP7A1 with respect to CEP170 shows not only proximal and overlapping patterns, but also distal patterns, in Figure 3 of our revised manuscript. Nevertheless, we agree with the review that the localization patterns of over-expressed DHCR7 and endogenous DHCR7 are not concordant. In response, we have generated new whole-cell over-expression data showing that the majority of over-expressed DHCR7 (Figure S1 D) and over-expressed CYP7A1 (Figure S3 B) localize away from the ciliary base. These patterns are seen, albeit to a lesser extent, for endogenous proteins as well. For instance, in the second row of Figure S1 A from our initial submission, endogenous DHCR7 can be seen in a peri-nuclear/ER pattern, consistent with the previously reported localization of exogenous DHCR7 (PMID 30535733). Interestingly, the different patterns of localization for endogenous and over-expressed DHCR7 and CYP7A1 proteins appear to be robust to transformation technique and antibody/tag pairs (Myc vs HA), as we have clarified in the revised Methods. Nevertheless, we also provide new data showing that over-expression of DHCR7 and CYP7A1 induce the Hedgehog transcriptional program (Figure S3 E). In sum, our results support the importance of focusing on endogenous protein localization and activity when studying mechanisms of Hedgehog signal transduction, as we have done in this study, and suggest that over-expression analyses may have contributed to the often confusing and unconcordant literature relating to DHCR7 function and Hedgehog pathway activity (PMID 26685159, 16687448, 31657721).

Introduction: Do Cyp7a1 and Hsb11b2 actually carry out the same reaction as stated? It appears the substrates are different, and the product of Cyp7a1 may be the 7-hydroxy rather than 7-keto form?

CYP7A1 and HSD11B2 both produce 7k-C, but do so using different substrates. We have clarified this point in the Introduction and Figure 5 F.

Fig 1F-H: Which exact sgRNA pair was used for these experiments? Can the authors quantify the results for Fig. 1G as in Fig. 2A (the other cells in Fig S1E don't appear to show as pronounced a decrease)?

We have generated new quantification of the immunofluorescence images from Figure 1 G (displayed in Figure 1 H of our revised manuscript) which validate that transduction of sgRNAs targeting *Dhcr7* reduced the intensity of DHCR7 at the ciliary base. Likewise, we have generated new data quantifying the immunofluorescence images from Figure 3 H (displayed in Figure 3 I of our revised manuscript) which validate that transduction of sgRNAs targeting *Cyp7a1* reduced the intensity of CYP7A1 at the ciliary base in response to Hedgehog pathway activation. The exact sgRNA pairs for these and other experiments have been stated in our revised Results and Methods.

Fig. 4A-B. What does it mean that Cyp7a1 knockdown reduced signaling in unstimulated cells? This is presumably a case where Cyp7a1 is not localized to the ciliary base.

In contrast to cholesterol, oxysterols such as 7k-C, 7k,27-OHC and 7b,27-DHC downstream of CYP7A1 activity are amphipathic and can diffuse throughout cells (PMID 12543708). Thus, freely diffusing oxysterols from CYP7A1 elsewhere in the cell (as we now show with new data in Figure S3 B of this revision) likely account for the decrease in Hedgehog transcriptional activity in unstimulated cells where *Cyp7a1* has been suppressed. We have clarified these data in the revised Results.

Fig. 4C-F: Which exact sgRNA was used? In some experiments, data are presented from one clonal CRISPRi knockdown. For select cases such as Fig. 4D-F, it would be more rigorous to show data from additional clones or sgRNAs.

We have revised the Figures, Figure Legends, and Methods to clearly denote which sgRNA sequences/cells were used for each experiment (including for Figure 4). Due to the experimental consistency among these unique reagents, not all monoclonal cell lines were used for every downstream experiment after validating gene suppression and the impact of gene suppression on gene product localization and Hedgehog signal transduction. For some sterolomic experiments, pooled results from multiple monoclonal cell lines are presented, as we have clarified in the Methods.

Fig. S3A: Are the panel labels or coloring wrong? It would be helpful to note in the text that Ptch1 KO MEFs were used.

Thank you for the careful evaluation of our supplementary data. We have clarified that *Ptch1*$^{-/-}$ MEFs were used for this experiment in the Results, and have corrected the coloring of the merged image.

Can the authors include the data for cholesterol levels related to Fig. 2H-J and comment on why they may be changed or not?

Thank you for bringing this important omission to our attention. We have generated new mass spectrometry-based sterolomics for cholesterol for this revision, and added these data to Figure S2 H, showing that *Ptch1* CRISPRi in NIH 3T3 cells, and genetic deletion of *Ptch1* in MEFs, does not alter the cellular levels of cholesterol compared to wild type cells. In contrast, we find that genetic deletion of *Ift88* in MEFs increases cellular levels of cholesterol compared to wild type MEFs. The absence of primary cilia in *Ift88*$^{-/-}$ MEFs (which, consequently, do not transduce ciliary Hedgehog signals) makes it challenging to interpret the increase in cellular cholesterol observed in these cells in the context of Hedgehog signaling. However, we show DHCR7 is removed from the ciliary base (Figure 2 C) and there is an increase in DHCR7 substrate (7-DHC) and substrate biproducts in *Ift88*$^{-/-}$ MEFs (Figure 2 J), consistent with a decrease in DHCR7 activity in *Ift88*$^{-/-}$ MEFs. In the context of equivalent DHCR7 transcript and protein levels after pharmacologic or genetic activation of the Hedgehog pathway (Fig. S2 A-G), the most likely explanation for the increase in cellular levels of cholesterol in *Ift88*$^{-/-}$ MEFs is compensatory activity from DHCR24, oxidosqualene cyclase, or other enzymes that participate in the cholesterol shunt pathway (PMID 12543708). These enzymes likely also contribute to the unchanged cellular levels of cholesterol in NIH 3T3 cells and MEFs after genetic inhibition of *Ptch1* (Figure S2 H). We have incorporated a description of these new data into our Results. In sum, these new data are consistent with the hypothesis (stated in our concluding paragraph) that tissue- or organism-specific expression of redundant enzymes participating in the production of Smoothened-activating sterols or oxysterols may influence Hedgehog signaling transduction.

Discussion: It would be worth noting that Cyp7a1 KO mice don't have an overt Hh phenotype (PMIDs 12588590 and 12093894).

We have incorporated this citation and a discussion of this mouse into the concluding paragraph of our manuscript.

September 25, 2020

Re: JCB manuscript #202002026R-A

Dr. David Ronan Raleigh
University of California San Francisco Medical Center
Radiation Oncology and Neurological Surgery
Helen Diller Family Cancer Research Building
1450 3rd Street, HD481
San Francisco, CA 94158

Dear Dr. Raleigh,

Thank you for submitting your revised manuscript entitled "Sterol and oxysterol synthases at the ciliary base activate the Hedgehog pathway" to Journal of Cell Biology. We sincerely apologize for the delay going through the re-review process. The manuscript has been assessed by the original reviewers, whose reports are appended below.

You will see that all three reviewers raised significant reservations about the revised manuscript. Some of these are important but addressable -- such as valid notes from Revs#1 and #2 about data presentation. However, other substantial concerns would require a lot more work. The reviewers were not convinced by some of the localization studies and by the revisions aimed at establishing the dependence on Smo. These are core issues that affect the central conclusions of the study. We have discussed these points in detail and considered the validity of the reviewers' arguments. Regrettably, we find ourselves sharing the reviewers' concerns at this stage.

As you know, JCB policy is that papers are considered through only one major revision cycle. We have discussed the paper in-depth, hoping to identify a path forward without major revision. However, we do not think that the manuscript can be published in JCB without major additional experimental revision. Given the significant remaining reviewer concerns and JCB policy, we unfortunately cannot offer publication of the manuscript. At this stage, we cannot go against the recommendations of three expert reviewers, whose comments reflect true scientific arguments and no signs of bias of any sorts. We unfortunately do not have enough reviewer support for publication. That leaves us with no other choice but to recommend that you submit this work elsewhere, in the interest of time, and consistent with journal policy.

Although we regret that we are not able to consider your manuscript further, we have discussed your manuscript with Life Science Alliance (http://www.life-science-alliance.org/) Executive Editor, Dr. Shachi Bhatt, and she would like to offer publication with minor textual changes. No further experimentation is needed for publication in LSA. LSA would encourage you to acknowledge the possibility of a Smo-independent pathway in DHCR7 regulation of Gli1 (as requested by Rev#2). Rev #3's concern about whether Dhcr7 and Cyp7a1 have local effects on cilia or global effects on the whole cell will be overruled for LSA. As for the concern about localization of Dhcr7 and Cyp7a1 in the ciliary pocket, LSA would request that you acknowledge the caveat of interpretation of the In-Out assay. Most other concerns raised by the referees should also be addressed by text changes/edits to the figures. Please do not hesitate to reach out to Shachi for discussions about transferring to LSA or with any questions.

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Link Not Available

If you would like to transfer your reviewer comments to another journal for consideration elsewhere, please contact the journal office and we would be happy to arrange the transfer on your behalf, cellbio@rockefeller.edu or call (212) 327-8588.

We appreciate the effort that has gone into the revisions and regret that the outcome is not more positive.

Sincerely,

Tamas Balla, MD, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This is a dramatically improved manuscript. However significant problems with the data and the interpretation remain.

1) Smo dependence of the process has not been adequately shown. Other mechanisms may be at play and more direct tests of Smo dependence are needed.
2) Figure 1K is presented as three separate experiments, which obscures the ability to see the effect of loss of Dhcr7 on basal Smo levels.
3) Figure 4C and D show that loss of Cyp7a1 reduces Smo accumulation in cilia after pathway activation, which is in contrast to the conclusions of the paper.
4) The localization of Dhcr7 to the ciliary pocket is not supported by the data. All that is really shown is that it is localized near the base of the cilium. The In/Out assay as used does not provide any information about what membrane Dhcr7 localizes to.
5) The localization Dhcr7 and Cyp7a1 to the ciliary base depends on the quality of the antibodies or to the localization of tagged constructs. The localization of the tagged constructs (S1D and S3B) is not convincing as I see no evidence of colocalization in the images. The endogenous protein localization looks good in the "representative" images (1G and 3H) but the quantification (1H and 3i) does not look so convincing.

Reviewer #2 (Comments to the Authors (Required)):

In their revised manuscript, "Sterol and oxysterol synthases at the ciliary base activate the Hedgehog pathway", the authors addressed many of the previous comments and improved aspects of the manuscript. However, there remain significant issues including some of the data not clearly supporting the assertions being made. The following need to be addressed:

1) Figure 1I appears to be the same graph as Figure 4E and is data from Cyp7a1 KD cells rather than Dhcr7 KD cells.

2) The logic surrounding the role of DHCR7 in Smo activation is not resolved.

The authors argue the following:
In Figure 1E, we see that sgDhcr7 decreases Gli1 expression in WT cells in the absence of SHH, and in Figure 1I (or what should be 1I) sgDhcr7 decreases Gli1 expression in the presence of SHH. So decreased Dhcr7 causes reduced Gli1 expression regardless of pathway stimulation.

In supplemental figure 3E we see that overexpression of Dhcr7 in WT cells is sufficient to increase Gli1 expression in the absence of SHH. And in Figure 1J we see that this effect of Dhcr7 overexpression is Smoothened dependent.

The authors go on to conclude that DHCR7 functions at the level of Smo activation. But data in Figure 1E do not agree with this conclusion, as loss of DHCR7 in wildtype Smo-expressing cells without SHH ligand reduces Gli1 transcription, suggesting that there may be a Smo-independent downstream function of DHCR7. Provided Gli1 transcription levels are different in sgDhcr7 cells and Smo-/- cells, this could be tested by introducing sgDhcr7 into Smo-/- cells and examining whether the sgDhcr7 Gli1 reduction is Smo dependent. At the very least, the authors must acknowledge their existing data are consistent with DHCR7 functioning downstream of Smo or in an alternate pathway.

3) There are issues with the new data in Figure 1K as well as its interpretation. The presentation of the data as three separate graphs is misleading. It appears the average for all untreated cells have been normalized to 1. These data are part of a single experiment, and therefore they should be grouped together and analyzed together.
The main issue with this analysis is that it does not address the original concern that DHCR7 KD could be affecting Smo dynamics in cilia, in either the on or off state. A similar data set in Figure 4C and D is presented in a more appropriate manner. In 4D the analysis shows that Cyp7a1 KD alone does not cause Smo to accumulate independent of SHH.
Presently, data in Figure 1K are not convincing and they do not support the assertion that DHCR7 specifically controls Smo activation, but not Smo accumulation. More appropriate analysis will uncover if DHCR7 KD alone is changing Smo dynamics. This would require a reorganization of the existing data to determine if there are differences between untreated dCas9-KRAB control and sgDhcr7 cells.
4) The authors conclude that sgCyp7a1 failed to block Smo accumulation in response to HH pathway activation with SHH, (Figure 4C and D). The representative image in 4C show the cilium of a SHH treated sgCYP7a1 cell without Smo present, which does not match this conclusion.

Moreover, there is some confusion as to what comparisons are considered significant. Is there no longer a difference in Smo accumulation between SHH treated control and sgCyp7a1 cells? The colors in 4D are confusing, are the significant differences highlighted made through comparisons with the untreated control group, or is either SHH treated group being compared back to its relative untreated control?

5) Minor error in the wording of Figure 1 legend for K.

Reviewer #3 (Comments to the Authors (Required)):

In their revised manuscript, Findakly et al. address questions raised by the reviewers and incorporate new data that further examine Dhcr7 and Cyp7a1 localization as well as the role of these enzymes in Smo trafficking. While some useful corrections and clarifications are made, on balance several of the key questions remain unanswered. Two of the main concerns raised previously were 1) the evidence presented showed that Dhcr7 and Cyp7a1 can modulate Hedgehog signaling but did not demonstrate that the pool of these enzymes at the ciliary base was functionally significant (or that signaling-induced changes in base localization influence signaling), and 2) while these enzymes appear to localize to the 'ciliary base', where exactly they are found within this area was not established, leaving it unclear exactly if/how they can alter the sterol composition of the ciliary membrane. The reviewed manuscript does not appear to contain new data that addresses the first of these points. The second point is addressed by analyzing localization of tagged Dhcr7 and Cyp7a1 and by using the In/Out assay to attempt to clarify Dhcr7 localization. However, as detailed below, these data only confuse the situation. Therefore, I stand by my original assessment that the model presented here is interesting and tackles an important unanswered question but is insufficiently supported by the data to warrant publication in JCB.

Major points:
1. In the absence of localized measurements of sterol levels at the cilium/ciliary base and in the absence of local perturbation of Dhcr7 or Cyp7a1 activity, it is not possible to distinguish whether these enzymes affect sterol metabolism and Hedgehog signaling through localized activity at the ciliary base versus their activity at the whole-cell level. While it is true that tools for localized lipid measurement or enzyme perturbation are not readily at hand, these technical difficulties don't obviate the importance of such information for demonstrating the authors' model. This point is underscored by evidence that the non-ciliary pools of these enzymes can modulate Hedgehog signaling: for example, Cyp7a1 CRISPRi reduces basal Hh signaling in unstimulated cells even though Cyp7a1 is not localized to the ciliary base under these conditions (Fig. 4A). One potential path forward would be to use a mutant form of Dhcr7 or Cyp7a1 that has defective localization to the ciliary base but normal activity at the whole-cell level. If such a mutant leads to altered Hedgehog signaling, that would support the authors' model; possibly the epitope-tagged forms of Dhcr7/Cyp7a1 would be useful for this as they fail to localize to the ciliary base.

2. The difficulty in showing a functional contribution of Dhcr7/Cyp7a1 at the cilium base places even more importance on the localization data. Here, I have concerns that the membrane structures at the ciliary base in which these enzymes are embedded are not defined, raising questions about if/how they modulate Hh signaling and about the reliability of the immunostaining results. In the case of Cyp7a1, I am not persuaded by the authors' rebuttal that Cyp7a1 is potentially not localized to membranes. TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) strongly predicts that Cyp7a1 is a transmembrane protein; little in PMID 24927729 (which the authors cite in their rebuttal) suggests otherwise ("We hypothesize that CYP7A1 is embedded into the outer leaflet of the membrane at a depth of approximately half of the lipid bilayer (~10-15 Å)."). Given that the apparent localization of Cyp7a1 does not appear to match that of known membranous structures at the ciliary base, its exact localization is an important unanswered question. Regarding Dhcr7, the In/Out assay data newly added in Fig. 5E does not show what the authors claim: that Dhcr7 is found in the ciliary pocket. Instead, it only shows that Dhcr7 is somewhere proximal to the 'outside' portion of the cilium. A similar result in Fig. 5E would be expected if Dhcr7 localized e.g. to the transition zone, inversin compartment, or any part of the basal body. In contrast, canonical pocket proteins such as Ehd1 exhibit an extended localization along the proximal portion of the cilium (PMID 25686250),

which is quite different from what is observed here for Dhcr7 (an additional minor point is that the HA-Arl13b construct is different from the pHluorin-Smo construct used in the original In/Out assay, and its topology and suitability for such experiments is not demonstrated).
A related concern is the reliability of the immunostaining results that show Dhcr7/Cyp7a1 localization to the base of the cilium. While new data is included based on HA-tagged forms of Dhcr7 and Cyp7a1, these proteins fail to localize to the base and do not match the reported localization of endogenous Dhcr7 and Cyp7a1. This could be due, as the authors suggest, to artefacts caused by over-expression or epitope-tagging. However, this discrepancy places even more importance on the performance and specificity of the Dhcr7 and Cyp7a1 antibodies used for immunostaining. The best support for these antibodies comes from the decreased ciliary base staining observed upon Dhcr7/Cyp7a1 CRISPRi. However, the new quantification of these signals in Figs. 1H and 3I shows that the signal is reduced by <50% upon CRISPRi. Validation by knockout would be much more compelling. Furthermore, are these modest changes in base localization upon enzyme knockdown responsible for the observed defects in Hedgehog signaling?

Other points
1. Regarding newly added Fig. S3 E, the HA-tagged Dhcr7 appears to be absent from the ciliary base (Fig S1D) and is instead primarily found in the cytoplasm and ER. This localization appears to differ both from the behavior of Myc-tagged Dhcr7 and from expectation, as this 9-pass transmembrane protein should not be observed in the cytoplasm. It is therefore not clear if this construct is functional. Also, if it can influence Hh signaling from outside the ciliary base, it is hard to see how this fits the authors' model of local regulation of sterol metabolism. Also, while the details are unclear, it appears this experiment was done in the absence of Hh pathway stimulation. If the authors' model is that Dhcr7 primes the ciliary microenvironment for Smo activation, how do they propose that Dhcr7-HA induces Gli1 expression in unstimulated cells where Smo is active?

2. The authors conclude that Dhcr7 and Cyp7a1 knockdown does not affect Smo ciliary accumulation. While this appears true for Dhcr7, in Fig. 4D Cyp7a1 CRISPRi cells appear to have decreased ciliary Smo in response to pathway stimulation.

Reviewer 2

1) Figure 1I appears to be the same graph as Figure 4E and is data from Cyp7a1 KD cells rather than Dhcr7 KD cells.

Thank you for bringing this error to our attention. We have re-linked the embedded .eps file in Fig. 1 l to the correct file displaying data from cells expressing sgRNAs suppressing *Dhcr7*, and corrected the PDF encoding Fig. 1 in our re-revised submission.

2) The logic surrounding the role of DHCR7 in Smo activation is not resolved. The authors argue the following: In Figure 1E, we see that sgDhcr7 decreases Gli1 expression in WT cells in the absence of SHH, and in Figure 1I (or what should be 1I) sgDhcr7 decreases Gli1 expression in the presence of SHH. So decreased Dhcr7 causes reduced Gli1 expression regardless of pathway stimulation. In supplemental figure 3E we see that overexpression of Dhcr7 in WT cells is sufficient to increase Gli1 expression in the absence of SHH. And in Figure 1J we see that this effect of Dhcr7 overexpression is Smoothened dependent. The authors go on to conclude that DHCR7 functions at the level of Smo activation. But data in Figure 1E do not agree with this conclusion, as loss of DHCR7 in wildtype Smo-expressing cells without SHH ligand reduces Gli1 transcription, suggesting that there may be a Smo-independent downstream function of DHCR7. Provided Gli1 transcription levels are different in sgDhcr7 cells and Smo-/- cells, this could be tested by introducing sgDhcr7 into Smo-/- cells and examining whether the sgDhcr7 Gli1 reduction is Smo dependent. At the very least, the authors must acknowledge their existing data are consistent with DHCR7 functioning downstream of Smo or in an alternate pathway.

We are grateful for this insightful interpretation of our data, which clearly necessitates clarification of our results in the context of published literature, and acknowledgement of alternative explanations for our findings. Thus, with respect to the primary concern of our interpretation of data presented Fig. 1 E, in our re-revised manuscript we now state “qRT-PCR for the Hedgehog target gene *Gli1* showed *Dhcr7* suppression inhibited basal Hedgehog signaling compared to control (Fig. 1 E), suggesting DHCR7 contributes to Hedgehog signal transduction even without pathway activation. Single-molecule imaging studies have identified Smoothened in cilia in the absence of pathway activation, and revealed Smoothened accumulation in cilia is associated with changing diffusion coefficient suggestive of interactions with proteins or lipids in the ciliary microenvironment (Weiss et al., 2019; Milenkovic et al., 2015). Although the absence transient activating interactions with the products of DHCR7 activity in cilia may account for the decrease in *Gli1* expression we observed after *Dhcr7* suppression (Fig. 1 E), it is also possible that DHCR7 may contribute to Hedgehog signal transduction downstream of Smoothened, or through non-canonical pathways.” With respect to the other concerns relating to our interpretation of data presented in Fig. 1 J and Fig. S3 E, we have taken this opportunity to also highlight the likelihood that other mechanisms (independent of Smoothened or ciliary Hedgehog signaling) may account for findings elsewhere in our re-revised manuscript.

3) There are issues with the new data in Figure 1K as well as its interpretation. The presentation of the data as three separate graphs is misleading. It appears the average for all untreated cells have been normalized to 1. These data are part of a single experiment, and therefore they should be grouped together and analyzed together. The main issue with this analysis is that it does not address the original concern that DHCR7 KD could be affecting Smo dynamics in cilia, in either the on or off state. A similar data set in Figure 4C and D is presented in a more appropriate manner. In 4D the analysis shows that Cyp7a1 KD alone does not cause Smo to accumulate independent of SHH. Presently, data in Figure 1K are not convincing and they do not support the assertion that DHCR7 specifically controls Smo activation, but not Smo accumulation. More appropriate analysis will uncover if DHCR7 KD alone is changing Smo dynamics. This would require a reorganization of the existing data to determine if there are differences between untreated dCas9-KRAB control and sgDhcr7 cells.

In our re-revised submission, we have re-normalized the data for all conditions in Fig. 1 K relative to control NIH 3T3$^{dCas9\text{-}KRAB}$ cells not expressing *Dhcr7* sgRNAs, and not treated with SHH. Further, we have grouped our data from all conditions together for re-analysis. Consistent with the lack of change in Smoothened accumulation in primary cilia after *Cyp7a1* suppression in Fig. 4 D, we now show in Fig. 1 K that *Dhcr7* suppression fails to alter Smoothened accumulation in primary cilia with or without Hedgehog pathway stimulation. Stated another way, *Dhcr7* suppression alone does not cause Smoothened to accumulate in cilia independent of SHH, nor does *Dhcr7* suppression alter Smoothened accumulation in cilia in response to SHH. These data are consistent with our findings for CYP7A1 in Fig. 4 D. In the context of our finding that Smoothened is required for DHCR7 to activate the Hedgehog transcriptional program in the absence of

pathway stimulation (Fig. 1 J), the clarified data in our re-revised manuscript "indicate DHCR7 contributes to Hedgehog pathway activation, but does not regulate Smoothened accumulation in primary cilia." More broadly, we have moderated the strength of our conclusions with respect to the mechanistic relationship between DHCR7 and Smoothened throughout our re-revised manuscript, focusing instead on the relationship between DHCR7 and Hedgehog pathway, and de-emphasizing the relationship between DHCR7 and Smoothened.

4) The authors conclude that sgCyp7a1 failed to block Smo accumulation in response to HH pathway activation with SHH, (Figure 4C and D). The representative image in 4C show the cilium of a SHH treated sgCYP7a1 cell without Smo present, which does not match this conclusion. Moreover, there is some confusion as to what comparisons are considered significant. Is there no longer a difference in Smo accumulation between SHH treated control and sgCyp7a1 cells? The colors in 4D are confusing, are the significant differences highlighted made through comparisons with the untreated control group, or is either SHH treated group being compared back to its relative untreated control?

We regret that the same linking error that is now corrected in Fig. 1 I (described above) also lead to a mistake in the .eps file embedded in Fig. 4 C. We have now corrected the panel corresponding to the cilium that received sg*Cyp7a1*/SHH treatment in Fig. 4 C, and stated in our re-revised text that "Consistent with suppression of *Dhcr7* (Fig. 1 K), quantitative immunofluorescence confocal microscopy revealed *Cyp7a1* suppression failed to block Smoothened accumulation in NIH 3T3$^{dCas9\text{-}KRAB}$ cilia in response to Hedgehog pathway activation with SHH (Fig. 4 C and D)." These results were statistically significant "compared to either control cells treated with vehicle, or cells expressing sg*Cyp7a1* treated with vehicle," as is now stated in the re-revised Figure Legend corresponding to Fig. 4 D. We have also taken this opportunity to highlight the similar statistically significant trends among conditions in the Figure Legend corresponding to Fig. 1 K (see point #3 above). Finally, we have re-colored the conditions in Fig. 4 D (and in Fig. 1 K) to clarify which conditions received sgRNAs (grey) and which conditions received SHH (filled compared to open circles). These changes are consistent with data presentation elsewhere in our manuscript. We thank the reviewer for their careful evaluation of our figure panels.

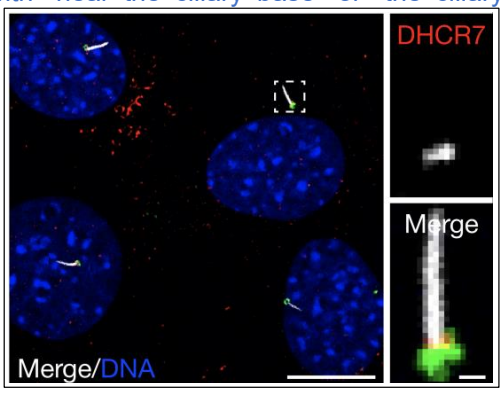
5) Minor error in the wording of Figure 1 legend for K.

We have corrected this error in our re-revised manuscript. Thank you for the careful reading of our manuscript and figure legends.

Editor

We strongly feel that toning down the conclusions from the localization studies is needed. None of the experts we consulted agreed that the data support a localization at the ciliary pocket. However, they felt that 'near the cilium base' was accurate. Therefore, we encourage you to revise the text accordingly. Our expert additionally had a question for you that we feel should be addressed in the text of the revised ms: the presence of the enzymes at the ER and Golgi, while mentioned in the text, is not visible on most images. If the enzymes are mostly detected only at the ciliary base, please indicate so, otherwise providing a couple of additional images (perhaps less adjusted) or describing them further would be helpful. This should not require new experimentation.

In the text of our revised manuscript, and in the titles of our revised figures, we have removed all references to "the ciliary base" and "the ciliary pocket" and replaced these with "near the ciliary base" or "the ciliary microenvironment." Moreover, we have revised our title to read "Sterol and oxysterol synthases near the ciliary base activate the Hedgehog pathway." We anticipate these changes will more accurately reflect the precision of our localization studies, but we welcome additional editorial input about our title and word choice elsewhere in the text of our manuscript.



With respect to the presence of endogenous enzymes away from the ciliary microenvironment, the best image of this is presented in Fig. S1 A (re-presented to the right, here). Although this image is perhaps most convincing for localization of an endogenous enzyme away from cilia, we are suspicious that much of this staining may be non-specific. Indeed, as is now stated in the

re-revised figure legend for Fig. S1 G, where we tested our antibodies in NIH 3T3$^{dCas9\text{-}KRAB}$ cells expressing sg*Dhcr7*, "note that speckled immunofluorescence staining away from the ciliary microenvironment does not attenuate with *sgDhcr7* transduction, suggestive of nonspecific staining." To eliminate the confusion generated by our prior sentences, we have re-revised the sentence previously mentioning ER and Golgi staining to read "We performed immunofluorescence confocal microscopy for DHCR7 in ciliated NIH 3T3 cells and found that endogenous DHCR7 predominantly localized near the ciliary base (Fig. 1 A and Fig. S1 A)." All our immunofluorescence images were adjusted using concurrent secondary-only controls for each experiment. Thus, we are confident our findings are not the product of over-adjustment. To clarify this important point, we have added the following sentence to the Microscope image acquisition and analysis" section of our re-revised Materials and Methods: "For presentation, image intensity was adjusted to suppress background staining from nonspecific interactions that were identified for each experiment using secondary only controls."

We agree with all the experts consulted that if the relationship to SMO is not fully defined, the text should be revised accordingly. In their opinion, the paper does not show convincingly that Smo is directly involved as a mediator between DHCR7 activity/relocalization and Hh pathway activation. Please follow the suggestions from Rev#2 in re-review for text revisions.

In response, we have removed all references to a potential functional relationship between DHCR7 and Smoothened in our abstract, and moderated all language related to the involvement of Smoothened for the mechanisms we study throughout our re-revised manuscript. For instance, with respect to our *Smo*$^{-/-}$ MEF data presented in Fig. 1 J, we now summarize our results by saying "These data suggest DHCR7 may contribute to Smoothened activation upon Hedgehog stimulation…" Further, we have more clearly delineated our data relating to activation of the Hedgehog transcriptional program from our data relating to Smoothened accumulation in primary cilia. For instance, with respect to our Smoothened localization studies in Fig. 1 K, we now summarize our results by saying "these data indicate DHCR7 contributes to Hedgehog pathway activation, but does not regulate Smoothened accumulation in primary cilia." We have taken this opportunity to also moderate our interpretations of the potential mechanistic relationship between CYP7A1 and Smoothened, revising the concluding sentence associated with Fig. 4 to read "In sum, these data demonstrate that CYP7A1 near the ciliary base promotes Hedgehog signaling."

We would only suggest resubmitting if you can fully and convincingly address Rev#2's point #3 from re-review. We would expect the results to be convincing for publication following re-analysis.

Please see our response to point #3 from Reviewer 2 above. Based on our email correspondence with the editorial staff, our interpretation of this comment was that a re-analysis and re-framing of our data were necessary to separate Smoothened/Hedgehog pathway activation, from Smoothened accumulation in primary cilia, in the context of DHCR7 activity. Thus, we have re-normalized and aggregated our data from control and *Dhcr7*-suppressed cells into a single graph in Fig. 1 K, demonstrating that DHCR7 does not regulate Smoothened accumulation in primary cilia.

We also routinely grant reasonable extensions in the word count for the paper (20K characters, excluding M&M and refs, which are unlimited), so please make text edits as needed.

Thank you for bringing this important information to our attention. We have extensively revised our manuscript, adding new interpretations of our findings in the context of published literature and re-analyzed data. We welcome additional editorial input about the clarity of our conclusions, and would be happy to add additional contextualization anywhere in our manuscript.

Outline of critiques and text edits addressing residual concerns raised by Reviewer 1 and Reviewer 3.

Reviewer 1 echoed comments made by Reviewer 2 related to the unclear mechanistic relationship between DHCR7 and Smoothened, and analysis of data in Fig. 1 K. We addressed these concerns in our responses to Reviewer 2 above. Reviewer 1 also raised concerns relating to the localization of DHCR7 at the ciliary pocket, which we addressed in our first response to the Editor in this section. Finally, Reviewer 1 expressed concerns about the discrepancies between endogenous and exogenous enzyme localization studies, which we address in our first response to the Advisor in the following section.

Reviewer 3 reiterated their initial concern for the absence of localized measurements of sterol levels at the cilium or ciliary base in our study. We addressed this issue in our initial response letter, citing technical barriers

that have hindered cell biology lipid research for decades. In the final paragraph of our re-revised manuscript, we now offer the following prose to highlight these limitations: “The list of enzymes participating in the production of Smoothened-activating lipids is growing (Kinnebrew et al., 2019), but our ability to understand mechanistic relationships between Hedgehog signaling and ciliary lipids depend on the emergence of new technologies to isolate primary cilia for mass spectrometry-based sterolomics. Indeed, an important limitation of our study is that our sterolomics were performed on whole cells. The genetic perturbations necessary to repeat these experiments in model systems where primary cilia can be isolated for biochemistry, such as *Stongylocentrotus purpuratus*, *Salpingoeca rosetta, Nematostella vectensis, Chlamydomonas reinhardtii,* or *Sus scrofa* cells, are not currently possible. Thus, significant technical advances are necessary to improve our understanding of Hedgehog signaling and ciliary lipids. Chief among these is likely the improvement of cellular probes for localizing cellular cholesterol (Courtney et al., 2018), which are not as refined as reagents available for studying phosphoinositides in primary cilia (Balla, 2013; Chávez et al., 2015; Garcia-Gonzalo et al., 2015).”

Reviewer 3 also reiterated their initial concerns relating to the previse localization of DHCR7 and CYP7A1 in the ciliary microenvironment. As described in our first response to the Editor in this section, we have moderated the language used to describe our localization studies, now using “near the ciliary base” and “ciliary microenvironment” throughout our re-revised manuscript. With respect to our interpretation of the IN/OUT assay, we offer the following to contextualize our results with respect to findings of canonical ciliary pocket proteins, such as EHD1: “These data suggest DHCR7 may localize to endosomes near the ciliary base, or perhaps at the nadir of the ciliary pocket, either of which may also be true for CYP7A1, a predicted single-pass transmembrane protein.”

Finally, Reviewer 3 expressed minor concerns related to (i) our exogenous enzyme localization studies, which we address in our first response to the Advisor in the following section, and (ii) the impact of DHCR7 and CYP7A1 on Smoothened localization, which we addressed in our response to Reviewer 2 (point 3) above.

Advisor

Fig. S1C and D) The two images can’t be compared since one image is an overview and the other just a small detail showing the cilium. An overview of the myc-tagged DHCR7 would be better.

This point is well taken. In the first paragraph of our re-revised Results and Discussion, we now state “over-expressed exogenous DHCR7 with a Myc tag could be found at the ciliary base in MEFs (Fig. S1 C). However, exogenous DHCR7 with an HA tag that was over-expressed in MEFs using a different transfection reagent localized to the cytoplasm and endoplasmic reticulum (Fig. S1 D), which was also the case for the majority of over-expressed DHCR7 with a Myc tag. These data are consistent with previous reports (Koczok et al., 2019), but contrast with our results for endogenous DHCR7, underscoring the importance of localization studies that focus on endogenous gene products, rather than over-expression of exogenous constructs that may be influenced by expression conditions or epitope tags.” We have taken this opportunity to also provide greater explanation for the discrepant subcellular localization of exogenous CYP7A1 constructs shown in Fig. S3 A and Fig. S3 B. Nevertheless, due to (i) formatting restrictions at rhe journal related to figure size, (ii) the fact that the mis-localization of exogenous constructs is a minor (albeit interesting) aspect of this manuscript, and (iii) the fact that Fig. S1 is already at the maximum allowable size, containing (1) whole cell images of endogenous DHCR7 with multiple basal body markers (Fig. S1 A), (2) whole cell images of endogenous DHCR7 using super-resolution microscopy (Fig. S1 B), (3) mass spectrometry-based sterolomics after CRISPRi suppression of *Dhcr7* (Fig. S1 E, F), and (4) whole cell images of endogenous DHCR7 after CRISPRi suppression of *Dhcr7* (Fig. S1 G), we have elected to retain our original whole cell images of exogenous DHCR7 with an HA tag (Fig. S1 D) and our partial cell images of exogenous DHCR7 with a Myc tag (Fig. S1 C). We are hopeful that the greater detail we now provide in the re-revised text of our manuscript (described above) will be sufficient to compare and contrast the results we observed with DHCR7 over-expression.

Fig. 2 A, B and G) It needs to be explained briefly why some of the fluorescence intensity values are negative. The M&M section states: “Fluorescence intensity was quantified by subtracting background intensity and normalizing to the average intensity within the control condition for each experiment.” So, the signal at the ciliary base was often below that of the background?

To clarify, signal intensity near the ciliary base was *sometimes* below that of background. Indeed, <5% of all our normalized fluorescence intensity values were less than zero. Nevertheless, to clarify this important aspect of our data analysis, we have added the following sentences to the “Microscope image acquisition and

analysis" portion of our Materials and Methods: "Fluorescence intensity was quantified by subtracting background intensity immediately adjacent to the region of interest, and normalizing the resulting intensity to the average intensity within the control condition for each experiment. In rare instances, background intensity immediately adjacent to the region of interest was higher than intensity in the region of interest, especially for conditions where DHCR7 or CYP7A1 were absent from the ciliary base. In these cells, image quantification yielded negative normalized intensities, which are interpreted as within the margin of error for fluorescence intensity quantification."

In Fig. 5C but not 5A, negative values are recorded for CYP7A1 signal intensity. What explains the huge differences in the signals? Fig, 3C and 5C, right panel look different.

In contrast to Fig. 5 A and Fig. 3 C, Fig. 5 C is the only data in our paper obtained in the presence of cycloheximide. Thus, these data suggest that the addition of cycloheximide in Fig. 5 C increased non-specific background labeling from our CYP7A1 antibodies. Nevertheless, the magnitude of difference between control and treatment conditions in Fig. 5 C were equivalent to other changes for CYP7A1 localization near the ciliary base displayed elsewhere in our manuscript.

Fig. 5D is not enough for the conclusion that the centriolar structure is critical for DHCR7 accumulation. As the signal at the base is somewhat variable, I recommend to provide some statistical analysis.

Thank you for this excellent suggestion. In response, we have quantified fluorescence intensity of DHCR7 at the centriole in wild type and *Ofd1*^{GT} mouse embryonic stem cells, and provided those data alongside statistical analysis in a revised version of Fig. 5 D. Further, as is now stated in the re-revised text of our manuscript, these new analyses reveal "that DHCR7 accumulation near the centriole in *Ofd1*^{Gt} cells was reduced compared to wild type mouse embryonic stem cells (Fig. 5 D). Thus, like ciliary structure (Fig. 2 D), centriole structure appears to be important for sterol synthase localization near the ciliary base." As denoted in the re-revised figure legend corresponding to Fig. 5 D, these results were highly significant with $p<0.0001$ (Student's t test).

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October 23, 2020

RE: JCB Manuscript #202002026RR-A

Dr. David Ronan Raleigh
University of California San Francisco Medical Center
Radiation Oncology and Neurological Surgery
Helen Diller Family Cancer Research Building
1450 3rd Street, HD481
San Francisco, CA 94158

Dear Dr. Raleigh,

Thank you for submitting your revised manuscript entitled "Sterol and oxysterol synthases near the ciliary base activate the Hedgehog pathway". As discussed during your appeal, while we all felt that the observations you report are noteworthy, we also felt that revisions were needed to address the remaining points from Reviewer #2, with which our editorial adviser agreed. We have now assessed the rebuttal letter and the revised manuscript and received input from Reviewer #2. We all independently came to the conclusion that the changes have significantly strengthened the manuscript - including in the depth of both discussion and interpretation. We are satisfied with the way you have addressed the remaining critiques and the changes made and appreciate your efforts to more accurately describe the data in the text. We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

1) Tables must be provided as stand-alone editable documents for publication and cannot be embedded in the M&M. Please convert to paragraph form or separate the tables from the M&M (p.14, p.17).

2) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.
Please indicate n/sample size/how many experiments the data are representative of: 1CEFHIJK, figure 2 all pooled data, 3CDGI, 4ABDE, 5ABCD, S1EF, S2ACEFH, S3CDE

3) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.
- For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features *even if described in other published work or gifted to you by other investigators*
- For all oligos (primers, guide RNAs, RNAi oligos, etc.), sequences must be provided (if made available to you from the manufacturer), including for negative controls.

- Please provide the species for all antibodies.
- Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

4) A summary paragraph of all supplemental material should appear at the end of the Materials and methods section.
- Please include one brief descriptive sentence per item.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

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Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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Tamas Balla, MD, PhD
Monitoring Editor, Journal of Cell Biology

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