JCB

Range of Shh signaling in adrenal gland is limited by membrane contact to cells with primary cilia

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Re: JCB manuscript #201910087

Dr. Ivona Mateska
Max Planck Institute of Molecular Cell Biology and Genetics
Pfotenhauerstraße 108
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Germany

Dear Dr. Mateska,

Thank you for submitting your manuscript entitled "Range of SHH signaling in adrenal gland is limited by membrane contact and presence of primary cilia". Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

You will see that the referees were positive about the quality of the work but felt that revisions are needed to streamline the paper, strengthen the conclusions, and address some of the questions left open by the data. Echoing editorial concerns from our evaluation of the work at submission, both Revs #1 and #2 commented on the lack of insight into the molecule mediating the repressive effects of the conditioned media from NCI-H295R cells on Hedgehog signaling (see experimental suggestions from Rev#1 in point #4 and questions from Rev#2 in point #1). Rev#1 asked if the supernatant from homogenized adrenal glands also inhibits Hedgehog signaling, Rev#2 asked if non-transformed adrenocortical cells release this inhibitory factor. Rev#1 additionally requested immunofluorescence or in situ hybridization for SHH in the adrenal gland to support the claim that short-range SHH signaling from direct cell-to-cell contact mediates HH signaling in the adrenal gland (point #5) and Revs#1 and #3 asked about the ciliation status of the cells (Rev#1 #8, Rev#3 point #2).

We discussed the reviews editorially and feel that the reviewers provided excellent, valid, and constructive suggestions. In particular, the first two referees make relevant and testable points regarding the nature of the inhibitory factor. We would welcome the opportunity to consider a revised manuscript that addresses the reviewers' remarks. Publication in JCB will, in particular, require reorganizing the paper for clarity and further characterizations of the inhibitory activity along the lines suggested by the referees.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. Our typical timeframe for revisions is three to four months; if submitted within this timeframe, novelty will not be reassessed. We would be open to resubmission at a later date; however, please note that priority and novelty would be reassessed.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Your manuscript may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Your manuscript may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Maxence Nachury, PhD
Monitoring Editor, Journal of Cell Biology

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

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

One of the major functions of Hedgehog signaling is the development and homeostasis of tissues and organs. Previous work from this laboratory and others has demonstrated both short-range membrane-associated and long-range lipoprotein mediated modulation of Hedgehog signaling. This manuscript offers some insight into how the adrenal gland regulates Hedgehog signaling. The authors demonstrate that adrenocortical Shh ligand co-fractionates with the lipoprotein marker APOA1, and this phenotype is recapitulated into their representative in vitro carcinoma cell line NCI-H295R. This suggests Shh-lipoproteins are produced within the adrenal gland and in NCI-H295R

cells. Intriguingly, the Shh-lipoproteins or other lipoproteins secreted into the medium by these cells seem to inhibit the Hedgehog pathway. This inhibition appears to occur at the level of SMO, or perhaps distal to SMO. In contrast, in co-culture experiments, membrane associated Shh activates the Hedgehog pathway.

Although this manuscript offers an intriguing insight into how Shh-lipoproteins and other lipoproteins may regulate Hedgehog signaling differently than membrane-associated Shh in the context of the adrenal gland, several weaknesses emerge throughout the manuscript. First, the authors do not convincingly demonstrate that the Hedgehog pathway regulation found in the adrenal gland is recapitulated by the carcinoma cell line NCI-H295R. Second, although the authors demonstrate conditioned media from NCI-H295R cells represses Hedgehog signaling, they offer no insight into the identity of the molecule responsible for their observation. Third, at times, the manuscript appeared non-cohesive, jumping between understanding how Hedgehog signaling operates in the adrenal gland to maintain tissue architecture versus how adrenocortical carcinoma cells do not ciliate nor respond to canonical Hedgehog signaling. The data are interesting, although the narrative is, perhaps, obscured in several areas by longer-than-needed explanations of nonpositive results (SHH distribution in mice fed normal vs high fat diet, Warburg-like state, HUVEX cells, and TGFb)… all of which should remain in supplemental data, but should be de-emphasized in the Results section. Since the novel finding in this manuscript is the regulation of Hedgehog signaling through lipoproteins and membrane-associated Shh, I would broadly recommend the authors to focus on this finding. Specific comments include:

1. At multiple points in the text, the authors note that SHH co-fractionates with FLOTILLIN1 and TSG101, exovesicle markers. Although this certainly may be possible, the lack of a clean separation between lipoprotein makers APOE and APOA1 in the fractionations makes this a difficult claim to make. I would recommend the authors limit their conclusion to SHH co-fractionating with lipoprotein makers alone or conduct further confirmatory studies to definitively conclude SHH-macrovesicles exist both in dissected adrenal glands and in the NCI-H295R cell line medium. Further, The authors conclude from biochemical fractionation and differential centrifugation that SHH exists in lipoprotein and exovesicle fractions in the adrenal gland, but it is unclear to what extent this finding may be a technical artifact of sample homogenization and their results should be confirm microscopically. The presence of SHH in LDL, HDL and soluble fraction of Figure 1E raises concern for the specificity of these techniques. Further, the inconclusive definition of how SHH is released from NCI-H295R cells in Figure 3 raises concern for how well these cells recapitulate in vivo adrenal biology.
(1) A major connection between the cell line model and mouse adrenal glands would be to demonstrate that the supernatant containing SHH-lipoproteins and other lipoproteins also inhibits HH-signaling in the 3T3 Shh-LIGHT2 cells and/or via RT-qPCR for Gli1.
(2) Important controls should be included in experiments presented in Figure 2. Notably, (1) serum-free cultured NCI-H295R cells have a dramatically different lipoprotein profile and (2) demonstrate that bovine/human/mouse serum alone has no SHH.
(3) Although the Shh-LIGHT2 cells are a robust and well-used cell line to measure Hedgehog pathway activity, it is possible that the unidentified compound inhibiting Hedgehog activity may be interfering with the luciferase assay. A control RT-qPCR should to confirm that NCI-Shh-Lpp inhibits Hedgehog signaling, and this observation is not dependent upon the luciferase assay.
(4) The data presented in Figure 4 are woefully insufficient to conclude that adrenal cells co-secrete an inhibitory molecule with SHH on lipoproteins. The findings that NCI-H295R conditioned media inhibits Hedgehog pathway activation from HEK-293T conditioned media or SAG could be more easily explained by chelation of SHH (from both NCI-H295R or HEK-293T cells) from additional molecules in the conditioned media from NCI-H295R cells, or non-specific effects that do not so much inhibit the Hedgehog pathway as they likely disrupt the overall health (or cilia?) of these cells.

This assertion, and the lack of data supporting it, represents the greatest weakness of the paper and draws into significant question the results presented in Figure 5. Moreover, the questionable lack of cilia in adrenal cortex cells would suggest that these cells would not be expected to respond to Hedgehog ligands irrespective of any co-secreted inhibitory molecules. With respect to the in vivo relevance of any of these findings, does the supernatant from homogenized adrenal glands also inhibit the Hedgehog pathway? For publication into JCB, the authors should consider mechanistic work to offer at least a clue to the identity of the compound in concentrated medium that is inhibiting Hedgehog pathway activity in a competitive fashion as seen in 4d/e. The following techniques followed by the similar assays already done by the authors may offer insight:
a. Size exclusion chromatography - size of molecule
b. Normal/ Reverse phase high pressure liquid chromatography - polarity of molecule
c. Ion exchange chromatography - acidic / basic nature of molecule
(5) The second, primary weakness of this manuscript is the lack of immunofluorescence or in situ hybridization for SHH in the adrenal gland, which is required to support the primary hypothesis that short-range SHH signaling from direct cell-to-cell contact mediates HH signaling in the adrenal gland.
(6) It is unclear why non-treated cells in Figure 5b show an insignificant decrease in Hedgehog pathway activation following addition of the inhibitory lipoproteins. The authors should consider repeating this experiment, especially considering what appears to be two clusters in the lipoprotein treated samples.
(7) The authors should consider moving Figure 6 and Figure 8 to supplemental information, as these findings confuse the story presented. I would recommend the authors spend more time focusing on the identity of the molecule in the conditioned media that is repressing Hedgehog signaling and confirming this molecule is present in the adrenal gland. At the very least, the non-quantitative PCR bands in Figure 6A and 6B should be replaced by immunoblots if the authors which to interrogate and contrast the expression of Hedgehog pathway components in adrenal model systems. Likewise, for the authors to conclude that TGFb induces Gli1 expression downstream of Gli2 expression, repeat experiments with immunoblots and cyclohexamide treatment to block translation are required.
(8) Are HCI-H295R cells ciliated? The data presented in Figure 6 suggest they are not, which could greatly impact interpretation of the Hedgehog signaling mechanism(s) in these cells. In Figure 7A and 7B, the authors attempt to answer this question... but state in one sentence that NCI-295R cells do not have cilia, but then state that they have a "few" cilia in the very next sentence. Do the adrenal tissues that transduce Hedgehog signals express primary cilia? In Figure 7C, the authors suggest that they do not due to lack of ARL13B expression, but additional ciliary markers should be investigated, especially since the confusing issue of ARL13B-devoid cilia in NCI-295R cells looms over the interpretation of in vivo data. The authors use HEK-293 and HeLa cells to validate their assertion that NCI-H295R cells uniquely secrete only a low concentration of SHH on lipoproteins, but neither HEK-293 or HeLa cells express primary cilia or transduce ciliary Hedgehog signals, which raises concern for the specificity of the mechanism the authors propose.

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

In this manuscript, Mateska et al. investigate Hedgehog signaling in the adrenal gland and the factors that control target cell responsiveness to Shh ligands. They find that adrenocortical cells release lipoprotein-associated Shh but that this Shh is co-secreted with a factor that inhibits paracrine Hedgehog signaling. Thus it is the membrane-associated Shh pool of adrenocortical cells that is able to stimulate downstream signaling, but only in immediately adjacent cells and not in

more distant cells or in an autocrine fashion (due to absence of cilia on the Shh-producing cells). Overall, the quality of the data are high, the conclusions are well supported, and the manuscript is well written and clearly organized. The findings are also likely to be of high interest to JCB's readership given their relevance to Hedgehog signaling, adrenal gland biology, and the spatial organization of morphogen signaling. As detailed below, there are a few issues, mostly minor in nature, that I believe should be addressed before the manuscript is suitable for publication.

Major point:
A key observation is that the lipoprotein-associated Shh released by adrenocortical carcinoma cells is co-secreted with a factor that inhibits Shh signaling, which is proposed to control the range of Shh signaling in vivo. The authors also suggest that this inhibitory factor is not secreted by HEK-293 or HeLa cells that release lipoprotein-associated Shh (Fig. 4D). However, HEK-293 and HeLa cells seem to release at least ~25-fold more Shh than NCI-H295R cells (Supp. Fig. 2). Since it appears that the concentrated conditioned media applied in Fig. 4D were normalized to contain equal amounts of Shh, then presumably the NCI-H295R-derived medium (and any inhibitory factor therein) would be >25-fold more concentrated than the HeLa-derived medium. Is it possible that this difference contributes to the observation that NCI-H295R medium inhibits Shh signaling but that HeLa medium does not? In other words, is the inhibitory factor likely to be a specific feature of NCI-H295R cells or common to many Shh-producing cell types? Similarly, is it possible to determine whether normal, non-cancerous adrenocortical cells also release this inhibitory factor?

Minor points:
Pg. 2. It would be more accurate to state that Smo activation leads to a change in the post-translational processing of Gli transcription factors rather than to their stabilization per se (in fact activated Gli proteins appear to be quite labile; see PMID 20360384 and 20154143).

Pg. 13. It appears that the co-secreted factor that inhibits the Shh pathway could act at the level of Smo, as the authors suggest, but it also seems possible it could act downstream of Smo.

Pg. 13. In Fig. 5C-D, Smo ciliary localization, a marker of pathway activation, is shown to increase as the number of co-cultured NCI-H295R cells is increased, leading the authors to suggest that NCI-H295R cells can stimulate Hedgehog signaling in cells they directly contact. Are the authors able to determine or quantify whether Smo ciliary accumulation specifically occurs in the NIH3T3 cells directly adjacent to NCI-H295R but not in NIH3T3 cells distant from NCI-H295R cells? Such data could clarify whether direct contact between Shh-producing and responding cells is necessary. Additionally, the Smo-mEos2+ positive structures in the right-most panel of Fig. 5D look a bit odd - are these definitely Arl13b+ cilia?

Pg. 14. It should be noted that other groups have reported that HUVEC and other endothelial cells can be ciliated (at least to some extent; see PMIDs 15024030, 26430510, 22001693, 24561257).

Pg. 17. The authors state that NCI-H295R cells do not have cilia or express Arl13b. It seems more accurate to state they do not have Arl13b-positive cilia, as Arl13b gene expression was not directly examined.

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

Summary

In their manuscript, "Range of SHH Signaling in Adrenal Gland is limited by membrane Contact and Presence of primary cilia", Mateska et al. seek to identify the role of SHH secretion in adrenal gland cells and cancer cell lines. In their model of SHH secretion they find that secreted SHH is bound to lipoproteins, but this form of SHH is inhibited by co-secreted factors, preventing long-range signaling. Therefore, only short range SHH signaling using membrane-bound ligand remains intact. In the receiving cell, this signaling requires a cilium, which the authors also show must stain positive for the cilia protein Arl13b. The issue of how SHH is secreted and travels to target cells is a longstanding one and this work contributes to identifying several relevant mechanisms. Identifying these mechanisms in the adrenal gland thus moves the field forward by defining them in relevant contexts. Furthermore, the authors show that adrenocortical carcinoma cells work around these mechanisms altogether which may be critical in understanding how to target activation of Shh targets in cancer. Overall, the data are well-controlled and appropriately interpreted but there are several remaining issues:

Major Concerns

1) The paper meanders from mechanism to mechanism and hard for the reader to follow as it is unclear where it is going. The reader would benefit from a road map at the beginning of the manuscript. As is, the manuscript jumps from exovesicular proteins, to lipoproteins, to cilia, and finally to Arl13b positive cilia. Due to this meandering, the proposed model at the end is a bit murky. While each experiment makes sense and the conclusions drawn are appropriate, the flow between them is hard to follow. Perhaps the authors could take the time to establish the potential models in the introduction before experimentally addressing each one.

2) The authors propose that Arl13b positive cilia are required for mediating SHH. There are several issues to address regarding this data.

a) If these carcinoma cell phenotypes are a functional result of the cilia being Arl13b negative, Smo and other SHH components would be predicted to be enriched. The authors demonstrate in Figure 6A that these cells express SHH components, including Smo, so this should be examined.

b) The ciliation of the distinct cell populations in the co-culture is critical and needs clarification. In figure 5, it is unclear how the authors separated NIH3T3-specific cilia from carcinoma cell cilia. The authors have yet to establish the ciliation rates of NIH3T3 and carcinoma lines. Furthermore, Figure 7B shows the carcinoma cells display some cilia so the authors need to explain how NIH3T3 verses carcinoma cell cilia were defined.

c) In Figure 7 panel C it is unclear that there are Arl13b negative cilia as the authors state. There are some acetylated □-tubulin positive puncta in the image shown, are there no cilia, or few cilia? Quantification of ciliation in vivo would be informative and support the model proposed in 7D.

d) Finally, the language describing the role of Arl13b in the SHH pathway should be amended as Arl13b is not a trafficking protein, but like most GTPases it influences the trafficking of membrane proteins. Arl13b is known to influence GliA and not GliR forms, this needs correcting in the discussion.

3) The authors should amend their discussion to include the role of Dispatched in cholesterolated Hh secretion. The Anderson lab previously showed that juxtracrine SHH signaling in the notochord of embryos lacking Dispatched maintains the notochord, which is relevant here as it represents membrane to membrane signaling.

4) In figure 1E, the representative blots are faint and difficult to interpret. In this panel it is not clear that higher density fractions are enriched with SHH as the authors claim. Moreover, this assertion is softened to a partial association with SHH in Figure 3, where the role of exovesicles is dismissed entirely. These inconsistencies are confusing and diminish clarity of the manuscript.

Minor Concerns

There are a few places where the description of the result precedes the explanation of the experiment. One example- on page 11 paragraph 2, the second sentence beginning with "We observed..." needs to come after the subsequent explanation of what was done. A proofreading for this will easily fix it.

The authors should clarify the differences between cell lines used in figure 5. The Shh-LIGHT2 cells are NIH3T3, but distinct from those used in co-cultured experiments. An explanation of why the Shh-LIGHT2 cells were not used for these co-culture experiments would also clarify the experimental details.

There is not a clear indicator of how many cells and cilia were examined in experiments shown in figures 5 and 7.

The font of the graphs of several figures is too small and difficult to read.

The x-axis labeling of Figure 5C is confusing. SAG treated control cells should be moved closer to the untreated controls.

Max Planck Institute of Molecular Cell Biology and Genetics

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Dresden, July 27, 2020

Dear Dr. Casadio, dear Dr. Nachury,

We are pleased with the general positive response from the editorial body and the reviewers on our manuscript and thank you for the opportunity to revise and resubmit it. We are very grateful to the reviewers for their constructive comments - we considered them very carefully and addressed as many of them as possible. In response to the comments, we conducted a series of additional experiments and have now added significant amount of new data in the manuscript (Figure 1A, 2C - D, 4F - K, 5B - C, 6C, 7D, 8B and Supplementary Figure 2B – D and 4). We also added additional data in the Response letter for further clarification of some of the reviewer's questions (Figures 1-3 'For reviewers only'). Additionally, we revised the manuscript to improve its flow and clarity. We believe that the results of the suggested experiments and analyses significantly strengthen our work and the initial conclusions.

Please find appended the point-by-point response to the reviewers addressing in detail their questions and comments. Changes in the revised manuscript are highlighted in yellow.

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

One of the major functions of Hedgehog signaling is the development and homeostasis of tissues and organs. Previous work from this laboratory and others has demonstrated both short-range membrane-associated and long-range lipoprotein mediated modulation of Hedgehog signaling. This manuscript offers some insight into how the adrenal gland regulates Hedgehog signaling. The authors demonstrate that adrenocortical Shh ligand co-fractionates with the lipoprotein marker APOA1, and this phenotype is recapitulated into their representative in vitro carcinoma cell line NCI-H295R. This suggests Shh-lipoproteins are produced within the adrenal gland and in NCI-H295R cells. Intriguingly, the Shh-lipoproteins or other lipoproteins secreted into the medium by these cells seem to inhibit the Hedgehog pathway. This inhibition appears to occur at the level of SMO, or perhaps distal to SMO. In contrast, in co-culture experiments, membrane associated Shh activates the Hedgehog pathway.
Although this manuscript offers an intriguing insight into how Shh-lipoproteins and other lipoproteins may regulate Hedgehog signaling differently than membrane-associated Shh in the context of the adrenal gland, several weaknesses emerge throughout the manuscript.

RESPONSE: We thank the reviewer for reading and commenting on our manuscript.

First, the authors do not convincingly demonstrate that the Hedgehog pathway regulation found in the adrenal gland is recapitulated by the carcinoma cell line NCI-H295R.

RESPONSE: To probe SHH secretion and signaling in this study we used the human adrenocortical carcinoma cell line NCI-H295R, which has been extensively used to study adrenal function and tumor biology (Gazdar et al. 1990; Rainey et al. 1994; 2004) and expresses *SHH* mRNA (Werminghaus et al. 2014). The low yield of primary adrenocortical cells from murine adrenal glands and the inability to culture these cells longer than 24 – 48 hours, rendered the use of primary adrenocortical cells in our assays impossible. Moreover, we were interested in comparing the Shh signaling in healthy adrenal gland and adrenocortical carcinoma cells.

Indeed, as pointed out by the reviewer, we found that the regulation of the Shh pathway in the murine adrenal glands and the NCI-H295R adrenocortical carcinoma cells is substantially different. Although both NCI-H295R and normal adrenocortical cells express Smo and Ptch1, the pathway is 'turned off' in the healthy adrenal gland, evidenced by absence of expression of the Shh transcriptional targets *Gli1* or *Gli2*, while in the NCI-H295R cells it is 'turned on', shown by constitutive high *GLI1* and *GLI2* expression (Figure 6 A – C). However, we show that this is not due to differences in Shh expression, since both NCI-H295R and normal murine adrenocortical cells efficiently produce SHH and secrete it in association with lipoproteins (Figure 1, Figure 2 and Figure 3). We found that both NCI-H295R and mouse adrenocortical cells lack ARL13B-positive cilia (Figure 7 A – D), important for responsiveness to the SHH ligand (Caspary et al. 2007; Larkins et al. 2011). The absence of ciliation could explain the non-responsiveness of NCI-H295R cells to the SHH ligand and the SMO agonist (Figure 6 D – F). Instead, similarly to other cancers (Dennler et al. 2007; Alexaki et al. 2010; Javelaud et al. 2011), we found that *GLI1* and *GLI2* can be triggered by TGF-β in NCI-H295R cells (Figure 8). In accordance, TGF-β is highly expressed in NCI-H295R cells while its expression is lower in the normal adrenocortical tissue (data not shown). Collectively, our data show that, with respect to SHH production and secretion, Shh autocrine signaling and ciliation, the NCI-H295R adrenocortical carcinoma cells resemble normal adrenocortical cells, but they have additionally acquired the ability to induce SHH target genes in response to TGF-β.

Second, although the authors demonstrate conditioned media from NCI-H295R cells represses Hedgehog signaling, they offer no insight into the identity of the molecule responsible for their observation.

RESPONSE: We have now conducted a series of experiments in an effort to identify the inhibitory molecule(s) present in the NCI-H295R conditioned media regulating Shh pathway activity. The results of these experiments are shown in Figure 4 H – K and demonstrate that:

- the inhibitory activity is retained by a 100 kDa filter, suggesting that the inhibitor(s) is a part of molecular complexes with a size greater than 100 kDa (Figure 4 H).
- the most potent inhibitory activity is within fractions of apparent molecular weight between 43 kDa and 660 kDa, as separated by gel filtration chromatography (Figure 4 I).

- inhibition of steroidogenesis with ketoconazole does not affect the inhibitory activity of NCI-H295R conditioned media (Figure 4 J).
- we identified the endocannabinoid-like molecules N-acyldopamine 18:1 and 20:4 as potential inhibitors of HEK-ShhNc activity (Figure 4 K).

Third, at times, the manuscript appeared non-cohesive, jumping between understanding how Hedgehog signaling operates in the adrenal gland to maintain tissue architecture versus how adrenocortical carcinoma cells do not ciliate nor respond to canonical Hedgehog signaling. The data are interesting, although the narrative is, perhaps, obscured in several areas by longer-than-needed explanations of nonpositive results (SHH distribution in mice fed normal vs high fat diet, Warburg-like state, HUVEC cells, and TGFb), all of which should remain in supplemental data, but should be de-emphasized in the Results section. Since the novel finding in this manuscript is the regulation of Hedgehog signaling through lipoproteins and membrane-associated Shh, I would broadly recommend the authors to focus on this finding.
RESPONSE: We thank the reviewer for this constructive comment on how to improve the manuscript. In the revised version of our paper we have reduced the parts referring to non-positive results, which are shown as supplementary data, for instance fractionation of SHH and lipoproteins in mice fed normal and high-fat diet (Supplementary Figure 1), non-canonical Shh signaling (Supplementary Figure 3) or data in HUVEC (Supplementary Figure 5). We have also revised the transitions between the different Results sections to improve the overall flow of the manuscript (see page 13 / line 4-5, page 14 / line 15-18, page 16 / line 16-19, page 17 / line 5-7 and 35-37).

Specific comments include:

1. At multiple points in the text, the authors note that SHH co-fractionates with FLOTILLIN1 and TSG101, exovesicle markers. Although this certainly may be possible, the lack of a clean separation between lipoprotein makers APOE and APOA1 in the fractionations makes this a difficult claim to make. I would recommend the authors limit their conclusion to SHH co-fractionating with lipoprotein makers alone or conduct further confirmatory studies to definitively conclude SHH-macrovesicles exist both in dissected adrenal glands and in the NCI-H295R cell line medium.
RESPONSE: Several groups have reported that the SHH protein can be associated with exovesicles released from cells (Tanaka et al. 2005; Vyas et al. 2014). Among the many different exosomal markers, we focused on FLOTILLIN1 and TSG101 which were shown to co-fractionate with SHH in supernatants from SHH-transfected HEK-293R cells (Vyas et al. 2014). However, since our immunoprecipitation results do not support the association of SHH with FLOTILLIN or TSG101, we followed the reviewer's recommendation and have now removed these data. Our conclusion is now limited to association of SHH with the apolipoproteins APOA1 and APOE (Figure 1 C – E, page 13 / line 13 and Figure 3 C – D, page 14 / line 7-9).

Further, the authors conclude from biochemical fractionation and differential centrifugation that SHH exists in lipoprotein and exovesicle fractions in the adrenal gland, but it is unclear to

what extent this finding may be a technical artifact of sample homogenization and their results should be confirmed microscopically. The presence of SHH in LDL, HDL and soluble fraction of Figure 1E raises concern for the specificity of these techniques.

RESPONSE: To investigate the form in which adrenocortical cells produce and secrete SHH we compared the distribution of SHH in density gradients to that of proteins with suggested roles in SHH secretion. It was previously shown that different fly and mammalian cell types release Hh/SHH on lipoproteins (Palm et al. 2013). Therefore, we focused on the association of SHH released from adrenocortical cells with the well-known lipoprotein markers, APOA1 and APOE. We homogenized murine adrenal glands in a high-salt buffer, known to prevent unspecific ionic interactions between the released SHH and cell membranes while maintaining SHH lipid modifications. This protocol was used previously to extract Hh from *Drosophila* wing imaginal disc tissue (Palm et al. 2013). To estimate the size of the carriers of released SHH, we subjected the adrenal homogenate to differential centrifugation to sequentially pellet particles of decreasing sizes. To characterize the density of these carriers, we fractionated each adrenal supernatant (S1, S16 and S150) by isopycnic gradient centrifugation, where particles separate solely based on their density. The presence of SHH in LDL, HDL and soluble fractions (as shown in Figure 1 D – E, Figure 2 E – G and Figure 3 C) implies that multiple distinct carriers might be involved in the release of SHH from the adrenal tissue, as previously described (Thérond, 2012).

Further, the inconclusive definition of how SHH is released from NCI-H295R cells in Figure 3 raises concern for how well these cells recapitulate in vivo adrenal biology.

RESPONSE: Our data suggest that APOA1- and APOE-positive lipoproteins act as SHH carriers in NCI-H295R cells (Figure 3 C – D). Nevertheless, it is certainly possible that SHH is also released on carriers other than lipoproteins, both in adrenal glands and from NCI-H295R cells. Identification of other potential SHH carriers in the adrenocortical cells merits further investigation.

(1) A major connection between the cell line model and mouse adrenal glands would be to demonstrate that the supernatant containing SHH-lipoproteins and other lipoproteins also inhibits HH-signaling in the 3T3 Shh-LIGHT2 cells and/or via RT-qPCR for Gli1.

RESPONSE: We thank the reviewer for proposing this experiment. To recapitulate the inhibitory activity of the NCI-H295R cells supernatants in the adrenal glands, we have now examined whether homogenates of mouse adrenal glands and supernatants from primary mouse adrenal cell cultures can inhibit Shh signaling in the Shh-LIGHT2 reporter assay. Both adrenal gland homogenates and primary adrenal cell culture supernatants inhibit HEK-ShhNc activity in a dose-dependent manner, suggesting that the inhibitory factor(s) is/are also released from normal adrenocortical cells. These new results are now included in Figure 4 F - G (page 15 / line 3-6).

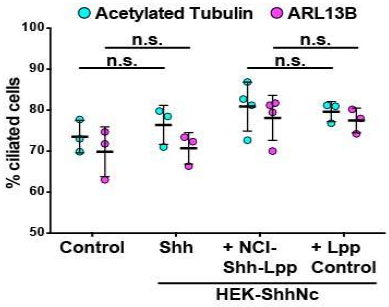
(2) Important controls should be included in experiments presented in Figure 2. Notably, (A) serum-free cultured NCI-H295R cells have a dramatically different lipoprotein profile and (B) demonstrate that bovine/human/mouse serum alone has no SHH.

RESPONSE: We thank the reviewer for suggesting these controls, which we have now included in Figure 2 C – D.
(A) We show that NCI-H295R cells produce and secrete lipoproteins, even in serum-free culture conditions. Western blot analysis of density gradient fractions from serum-free NCI-H295R-conditioned medium shows APOA1 in fractions with density of LDL and HDL (Figure 2 D). These lipoproteins, however, are not sufficient to induce SHH secretion into the medium, since no SHH was detected in serum-free NCI-H295R-conditioned medium (Figure 2 B, D; page 13 / line 32-33).
(B) We analyzed bovine, mouse and human serum by western blot and show that these sera do not contain SHH. On the same gel we loaded NCI-H295R-coditioned medium as a positive control, which contains a high amount of SHH (Figure 2 C; page 13 / line 30-31).

(3) Although the Shh-LIGHT2 cells are a robust and well-used cell line to measure Hedgehog pathway activity, it is possible that the unidentified compound inhibiting Hedgehog activity may be interfering with the luciferase assay. A control RT-qPCR should to confirm that NCI-Shh-Lpp inhibits Hedgehog signaling, and this observation is not dependent upon the luciferase assay.
RESPONSE: We have now measured *Gli1* expression in Shh-LIGHT2 cells treated with 10 ng HEK-ShhNc together with increasing amounts of NCI-H295R conditioned medium (NCI-Shh-Lpp) or corresponding controls. Treatment of Shh-LIGHT2 cells with NCI-H295R-conditioned media causes a dose-dependent reduction of the *Gli1* expression induced by HEK-derived ShhNc (Supplementary Figure 2 C). Hence, these data confirm the validity of the luciferase assay as a readout for Shh pathway activity.

(4) The data presented in Figure 4 are woefully insufficient to conclude that adrenal cells co-secrete an inhibitory molecule with SHH on lipoproteins. The findings that NCI-H295R conditioned media inhibits Hedgehog pathway activation from HEK-293T conditioned media or SAG could be more easily explained by chelation of SHH (from both NCI-H295R or HEK-293T cells) from additional molecules in the conditioned media from NCI-H295R cells, or non-specific effects that do not so much inhibit the Hedgehog pathway as they likely disrupt the overall health (or cilia?) of these cells. This assertion, and the lack of data supporting it, represents the greatest weakness of the paper and draws into significant question the results presented in Figure 5.
RESPONSE: As correctly pointed out by the reviewer, we cannot exclude the potential chelation of HEK-ShhNc from molecules in the NCI-H295R-conditioned medium. However, the demonstrated inhibitory behavior is specific to the NCI-H295R-conditioned medium and not to the HeLa-conditioned medium (Supplementary Figure 2 D). Moreover, the inhibition occurs downstream of SHH, at the level of SMO (Figure 4 E), which should not be affected by chelation of the SHH ligand. Therefore, we assume that the Hh pathway inhibition is due to a specific molecule(s) present in the NCI-H295R-conditioned medium rather than SHH chelation.
We monitored the effect of the NCI-H295R-conditioned medium on the overall health and viability of Shh-LIGHT2 cells in three ways. Shh-LIGHT2 cells constitutively express *Renilla luciferase* under the

Herpes simplex virus thymidine kinase promoter, providing constitutive low expression of Renilla luciferase enzyme as an internal control (Sasaki et al. 1997; Taipale et al. 2000). Treatment with NCI-H295R-conditioned medium does not alter the values of the Renilla luciferase activity, indicating that the NCI-H295R-derived supernatant does not affect the viability and overall health of Shh-LIGHT2 cells. Accordingly, the cell morphology inspected under light microscope at the end of each treatment is not affected. Lastly, we quantified ciliation in Shh-LIGHT2 cells treated with NCI-H295R-conditioned medium and saw no difference in the percentage of acetylated tubulin and ARL13B-positive cells between the control and treated samples (Figure 1_for Reviewers only). We added this information on page 14 / line 34-35.



AcTUB & ARL13B	475	561	484	469

Figure 1 for Reviewers only: Quantification of the percentage of ciliated Shh-LIGHT2, treated with 10 ng HEK-ShhNc alone or together with NCI-H295R-conditioned medium (NCI-Shh-Lpp) or Lpp control, counted as cells positive for AcTUB or ARL13B. The number of counted cells is given under the graph. N ≥ 3 replicates, presented as mean ± SD. Mann-Whitney test.

Moreover, the questionable lack of cilia in adrenal cortex cells would suggest that these cells would not be expected to respond to Hedgehog ligands irrespective of any co-secreted inhibitory molecules.

RESPONSE: This is correct. We did not detect *Gli1* and *Gli2* mRNA and protein expression in the murine adrenal cortex, suggesting that the Shh pathway there is inactive (Figure 6 A and C). Indeed, we assume that this is due to the lack of proper ciliation in the adrenal gland cortex. However, we assayed the SHH activity in Shh-LIGHT2 fibroblasts, which are ciliated, and show that they do not respond to SHH derived from HEK-293 cells in the presence of NCI-H295R-conditioned medium (Figure 4 D).

With respect to the in vivo relevance of any of these findings, does the supernatant from homogenized adrenal glands also inhibit the Hedgehog pathway?

RESPONSE: As explained above, homogenates of mouse adrenal glands and supernatants from primary mouse adrenal cell cultures also inhibit HEK-ShhNc activity (Figure 4 F – G).

For publication into JCB, the authors should consider mechanistic work to offer at least a clue to the identity of the compound in concentrated medium that is inhibiting Hedgehog pathway

activity in a competitive fashion as seen in 4d/e. The following techniques followed by the similar assays already done by the authors may offer insight:

a. Size exclusion chromatography - size of molecule

b. Normal/ Reverse phase high pressure liquid chromatography - polarity of molecule

c. Ion exchange chromatography - acidic / basic nature of molecule

RESPONSE: We agree with the reviewer that identification of the Shh pathway inhibitor(s) is an important question. We have now performed a series of assays to provide cues to the nature of the inhibitor. However, a complete biochemical analysis, as suggested by the reviewer, would require significant additional investment and, from our point of view, is beyond the scope of the paper.

We report the results of the experiments characterizing the nature of the Shh pathway inhibitory molecule(s) present in the NCI-H295R-conditioned media in Figure 4 H - K of the revised manuscript. They include:

- gel filtration chromatography and passage through filters of varying sizes (10 – 100 kDa) to provide insight on the size of the inhibitor. We found that the inhibitory activity is linked to molecule(s) or molecular complexes with an estimated molecular weight above 43 kDa, suggesting that the active molecule(s) can travel with lipoproteins (Figure 4 H - I, page 15 / line 10-15).
- inhibition of steroidogenesis in NCI-H295R cells, which showed that the inhibitory molecule(s) is not a steroid hormone (Figure 4 J, page 15 / line 15-18).
- testing the inhibitory activity of 18 different endocannabinoid lipids, out of which N-acyldopamine 18:1 and 20:4 were found to inhibit the activity of HEK-ShhNc (Figure 4 K, page 15 / line 19-24).
- LC-MRM analysis of supernatants from mouse adrenal glands detected dopamine and arachidonic acid (data not shown), the likely precursors of the inhibitory N-acyldopamine 20:4 (page 18 / line 31-35).

(5) The second, primary weakness of this manuscript is the lack of immunofluorescence or in situ hybridization for SHH in the adrenal gland, which is required to support the primary hypothesis that short-range SHH signaling from direct cell-to-cell contact mediates HH signaling in the adrenal gland.

RESPONSE: The specific localization of the Shh pathway components in the developing and adult murine adrenal gland has been previously described by several groups (Ching and Vilain 2009; King et al. 2009; Huang et al. 2010; Guasti et al. 2011; Wood and Hammer 2011; Laufer et al. 2012; Freedman et al. 2013) and we refer to these findings in the Introduction section (page 2 / line 39-40 - page 3 / line 1-8). In support, we now include an immunofluorescent staining of adrenal glands from Shh^{GFP} mice, showing the presence of SHH in the subcapsular cells, and from $Gli1^{LacZ}$ reporter mice, showing *Gli1* expression in the adrenal capsule (edited panel in Figure 1 A). These images confirm that the cells of the outer steroidogenic adrenal cortex produce SHH and the overlaying mesenchymal capsule cells respond by expressing *Gli1*.

(6) It is unclear why non-treated cells in Figure 5b show an insignificant decrease in Hedgehog pathway activation following addition of the inhibitory lipoproteins. The authors

should consider repeating this experiment, especially considering what appears to be two clusters in the lipoprotein treated samples.

RESPONSE: We have repeated this experiment three more times, running the Shh-LIGHT2 assay in duplicates for each condition. Taking all data together, the Gli1-dependent transcriptional activity in Shh-LIGHT2 cells co-cultured with NCI-H295R cells in serum-free conditions is significantly higher than in lipoprotein-supplemented cultures (Figure 5 B, page 16 / line 1-2).

(7) The authors should consider moving Figure 6 and Figure 8 to supplemental information, as these findings confuse the story presented. I would recommend the authors spend more time focusing on the identity of the molecule in the conditioned media that is repressing Hedgehog signaling and confirming this molecule is present in the adrenal gland. At the very least, the non-quantitative PCR bands in Figure 6A and 6B should be replaced by immunoblots if the authors wish to interrogate and contrast the expression of Hedgehog pathway components in adrenal model systems.

RESPONSE: We believe that the data presented in Figure 6 and Figure 8 provide important novel information for how the Shh pathway is regulated. We show that the Shh pathway is inactive in the healthy adrenal gland cortex, evidenced by the absence of *Gli1* and *Gli2* expression (Figure 6 A, C), demonstrating that there is specificity in the Shh signaling pattern. In contrast, adrenocortical carcinoma cells express *GLI1* and *GLI2* (Figures 6 B, C), indicating that they have evaded the regulatory control mechanisms that ensures specificity of Shh pathway in the adrenal gland. Answering how they do so is an important step in understanding what limits the specificity of Shh signaling in the adrenal gland and how that regulation may go awry in cancer. We show that these carcinoma cells upregulate SHH targets not by acquiring the ability to respond to SHH (they are poorly ciliated) but through a crosstalk with the TGF-β pathway (Figure 8).

To improve the overall flow and logic of the story, including the data shown in Figure 6 and Figure 8, we have modified the transitions between sections of the results (page 16 / line 16-19 and page 17 / line 35-37). In addition, we now provide immunoblots for Shh pathway components in adrenocortical carcinoma cells and mouse adrenal glands (Figure 6 C), which corroborate the mRNA expression data showing that GLI1, GLI2 and GLI3 are present in adrenocortical cancer cells, but not in healthy adrenal gland tissue (Figure 6 A - B).

Likewise, for the authors to conclude that TGFb induces Gli1 expression downstream of Gli2 expression, repeat experiments with immunoblots and cyclohexamide treatment to block translation are required.

RESPONSE: The observation that GLI1 expression is downstream of GLI2 was made in other cancers (Dennler et al. 2007). In the manuscript we state that in NCI-H295R cells TGF-β rapidly increases *GLI2* expression (already at 4 hours), followed by delayed increase of *GLI1* expression (at 24 hours) (Figure 8 A). We do not tackle the question whether induction of *GLI1* expression by TGF-β lies downstream of GLI2 translation, since we think that this is out of the scope of the present paper. However, as requested by the reviewer, we now show an increase in GLI2 and GLI1 protein levels in

TGF-β - treated NCI-H295R cells, which is abrogated by the protein synthesis inhibitor cycloheximide (Figure 8 B, page 18 / line 3-4), indicating that it requires protein translation.

(8) Are NCI-H295R cells ciliated? The data presented in Figure 6 suggest they are not, which could greatly impact interpretation of the Hedgehog signaling mechanism(s) in these cells. In Figure 7A and 7B, the authors attempt to answer this question... but state in one sentence that NCI-295R cells do not have cilia, but then state that they have a "few" cilia in the very next sentence.

RESPONSE: We thank the reviewer for noticing this inconsistency. Our quantification of cilia in NCI-H295R cells showed that 1 – 5% of NCI-H295R cells are positive for the ciliary marker acetylated tubulin, but only 0.5 – 2% of these cells are positive for ARL13B (Figure 7 A - B). Thus, we hypothesize that the inability of NCI-H295R to respond to SHH (Figure 6 E - F) is due to both their poor ciliation status and the lack of ARL13B protein in most of the present cilia. We have clarified this in the text accordingly (page 17 / line 9-11).

Do the adrenal tissues that transduce Hedgehog signals express primary cilia? In Figure 7C, the authors suggest that they do not due to lack of ARL13B expression, but additional ciliary markers should be investigated, especially since the confusing issue of ARL13B-devoid cilia in NCI-295R cells looms over the interpretation of in vivo data.

RESPONSE: We have now quantified the ciliation in the adrenal cortex (SHH-producing) and adrenal capsule (SHH-responding) cells and report these data in Figure 7 D. The quantification demonstrates that adrenocortical cells (SHH-producing) have fewer ARL13B-positive primary cilia compared to the overlaying capsule cells (SHH-receiving), in which ARL13B-positive primary cilia are abundant (Figure 7 C - D; page 17 / line 15-18). This result is consistent with the fact that the SHH derived from the cortical cells signals to the ciliated capsular cells, which respond by expressing the target gene *Gli1* ((King et al. 2009), and scheme in Figure 7 E). As requested by the reviewer, we also tested two additional ciliary markers: glutamylated tubulin (Bré et al. 1994; Lee et al. 2012) and IFT88 (Robert et al. 2007; Gigante et al. 2020) in NCI-H295R and NIH3T3/Smo-mEos2 cells. Both markers co-localize with acetylated tubulin in the primary cilia. Only a small fraction (1 - 5 %) of adrenocortical NCI-H295R cells are positive for glutamylated tubulin or IFT88, confirming our data that the majority of adrenocortical cells are not ciliated. In contrast, glutamylated tubulin and IFT88 are abundantly present in NIH3T3/Smo-mEos2 cells (these data are shown in Supplementary Figure 4).

The authors use HEK-293 and HeLa cells to validate their assertion that NCI-H295R cells uniquely secrete only a low concentration of SHH on lipoproteins, but neither HEK-293 or HeLa cells express primary cilia or transduce ciliary Hedgehog signals, which raises concern for the specificity of the mechanism the authors propose.

RESPONSE: The ability of a cell to produce and secrete SHH is independent of the presence of primary cilia on that cell. For example, the adrenocortical carcinoma cell line NCI-H295R produce SHH but lack adequate ciliation to respond to the SHH signal. However, in our studies, we used SHH-transfected HEK-293 and HeLa cells only as a means for production of the SHH ligand (Palm et al.

2013), but we tested its signaling activity in NIH3T3 cells which are highly ciliated and are known to respond to SHH (Milenkovic et al. 2009; Palm et al. 2013; Kim et al. 2014; Rodgers et al. 2016; Tukachinsky et al. 2016).

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

In this manuscript, Mateska et al. investigate Hedgehog signaling in the adrenal gland and the factors that control target cell responsiveness to Shh ligands. They find that adrenocortical cells release lipoprotein-associated Shh but that this Shh is co-secreted with a factor that inhibits paracrine Hedgehog signaling. Thus, it is the membrane-associated Shh pool of adrenocortical cells that is able to stimulate downstream signaling, but only in immediately adjacent cells and not in more distant cells or in an autocrine fashion (due to absence of cilia on the Shh-producing cells). Overall, the quality of the data are high, the conclusions are well supported, and the manuscript is well written and clearly organized. The findings are also likely to be of high interest to JCB's readership given their relevance to Hedgehog signaling, adrenal gland biology, and the spatial organization of morphogen signaling. As detailed below, there are a few issues, mostly minor in nature, that I believe should be addressed before the manuscript is suitable for publication.

RESPONSE: We thank the reviewer for these positive comments.

Major point:

A key observation is that the lipoprotein-associated Shh released by adrenocortical carcinoma cells is co-secreted with a factor that inhibits Shh signaling, which is proposed to control the range of Shh signaling in vivo. The authors also suggest that this inhibitory factor is not secreted by HEK-293 or HeLa cells that release lipoprotein-associated Shh (Fig. 4D). However, HEK-293 and HeLa cells seem to release at least ~25-fold more Shh than NCI-H295R cells (Supp. Fig. 2). Since it appears that the concentrated conditioned media applied in Fig. 4D were normalized to contain equal amounts of Shh, then presumably the NCI-H295R-derived medium (and any inhibitory factor therein) would be >25-fold more concentrated than the HeLa-derived medium. Is it possible that this difference contributes to the observation that NCI-H295R medium inhibits Shh signaling but that HeLa medium does not? In other words, is the inhibitory factor likely to be a specific feature of NCI-H295R cells or common to many Shh-producing cell types?

RESPONSE: We thank the reviewer for this insightful observation. To rule out the possibility that the concentration of the NCI-H295R-derived medium contributes to its inhibitory properties, and thus prove that the inhibitory activity is a specific feature of NCI-H295R cells, we tested in parallel NCI-H295R-conditioned medium and equally concentrated medium from HeLa cells, which do not produce SHH, for its inhibition of HEK-ShhNc activity. These results are presented in Supplementary Figure 2 D and show that, while the NCI-H295R-conditioned medium inhibits HEK-ShhNc activity in a dose-dependent manner, HeLa-conditioned medium does not (page 14 / line 35-38). These results show

that the inhibitory property is a specific characteristic of the NCI-H295R-derived conditioned medium. We should point out that concentration of the NCI-H295R-conditioned medium is required for inhibition of HEK-ShhNc activity (Supplementary Figure 2 B).

Similarly, is it possible to determine whether normal, non-cancerous adrenocortical cells also release this inhibitory factor?

RESPONSE: This is a very important point. We have now tested whether HEK-ShhNc activity can be inhibited by homogenates of mouse adrenal glands and by supernatants from primary mouse adrenal cell cultures. In both cases, we see a dose-dependent inhibition of HEK-ShhNc activity, suggesting that the inhibitory factor is also released from normal adrenocortical cells. These new results are now included in Figure 4 F-G (page 15 / line 3-6).

Minor points:

Pg. 2. It would be more accurate to state that Smo activation leads to a change in the post-translational processing of Gli transcription factors rather than to their stabilization per se (in fact activated Gli proteins appear to be quite labile; see PMID 20360384 and 20154143).

RESPONSE: The wording is now changed in the text (page 2 / line 20-21).

Pg. 13. It appears that the co-secreted factor that inhibits the Shh pathway could act at the level of Smo, as the authors suggest, but it also seems possible it could act downstream of Smo.

RESPONSE: It is a valid point that the inhibition of the Shh pathway by the NCI-H295R-secreted factor could also occur downstream of SMO. We now edited the text accordingly (page 15 / line 8-9).

Pg. 13. In Fig. 5C-D, Smo ciliary localization, a marker of pathway activation, is shown to increase as the number of co-cultured NCI-H295R cells is increased, leading the authors to suggest that NCI-H295R cells can stimulate Hedgehog signaling in cells they directly contact. Are the authors able to determine or quantify whether Smo ciliary accumulation specifically occurs in the NIH3T3 cells directly adjacent to NCI-H295R but not in NIH3T3 cells distant from NCI-H295R cells? Such data could clarify whether direct contact between Shh-producing and responding cells is necessary.

RESPONSE: We thank the reviewer for this suggestion. We now provide additional data to support our claim that direct contact between SHH-producing and SHH-responding cells is necessary for inducing signaling activity (new panel Figure 5 C). We cultured NCI-H295R cells and Shh-LIGHT2 cells in wall-separated culture inserts, where cells are not in direct physical contact but share the same culture medium. In such culture conditions, the Shh pathway in Shh-LIGHT2 cells would be induced only by the secreted lipoprotein-associated SHH from NCI-H295R cells. However, we see that Gli1-dependent luciferase activity increases only when NCI-H295R cells are cultured adjacent to Shh-LIGHT2 cells (Figure 5 C), implying that direct contact between SHH-producing and SHH-responding cells is indeed necessary for signal transduction (page 16 / line 2-4).

Additionally, the Smo-mEos2+ positive structures in the right-most panel of Fig. 5D look a bit odd - are these definitely Arl13b+ cilia?

RESPONSE: To clarify this issue, we provide here the separate channels for the panel 5D in question. Cilia are labeled with ARL13B in magenta, Smo-mEos2-positive cilia are green and the steroidogenic marker StAR labelling NCI-H295R cells is shown in red (Figure 2_for Reviewers only).

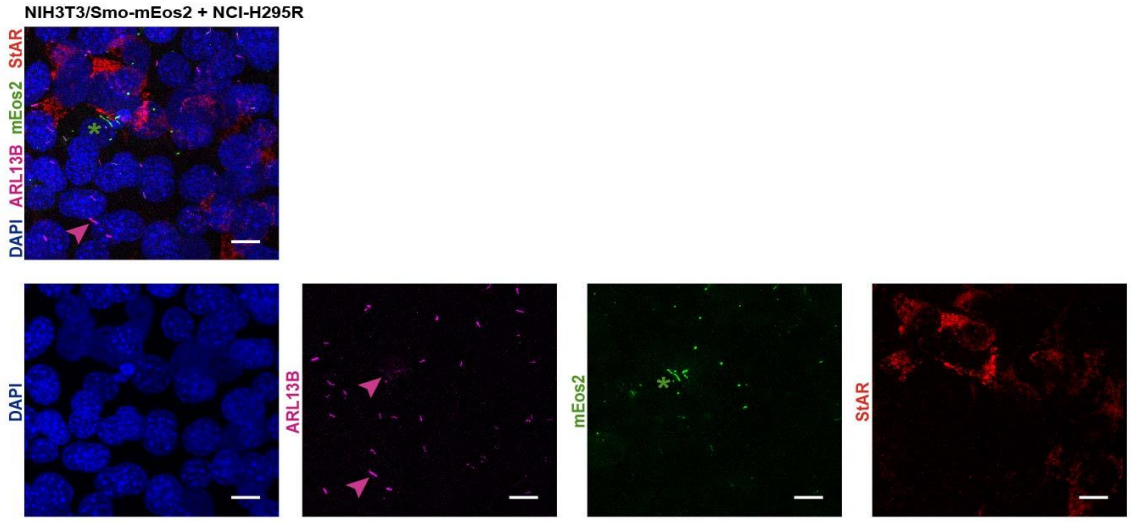


Figure 2_for Reviewers only: Immunofluorescence of 40,000 NIH3T3/Smo-mEos2 cells co-cultured with 40,000 NCI-H295R cells, labelled for ARL13B – cilia (magenta), Smo-mEos2 (green), StAR – a steroidogenic marker present in mitochondria of NCI-H295R cells (red) and nuclear DAPI (blue). ARL13B-positive cilia are denoted with magenta arrowheads. Ciliary SMO enrichment is indicated with an asterisk. Scale bar = 10 μm. The individual channels are shown in the lower panels.

Pg. 14. It should be noted that other groups have reported that HUVEC and other endothelial cells can be ciliated (at least to some extent; see PMIDs 15024030, 26430510, 22001693, 24561257).

RESPONSE: Different groups have indeed reported greatly variable cilia incidence on endothelial cells and specifically HUVEC. These studies examined cilia in different types of endothelial cells from different passages, cultured to either confluent or cobblestone stage and in different culture media. For example, (Wheatley et al. 1996) stated that endothelial cells from dog and rabbit as well as cultured HUVEC do not possess primary cilia. Geerts et al. cultured HUVEC in media with 2 % FBS and EGM-2 and reported up to 60 % ciliation at 12 days cobblestone stage, but less than 1 % cilia at confluence (Geerts et al. 2011). Iomini et al., on the other hand, cultured HUVEC with 20 % FBS and reported that 5 to 35 % HUVEC have primary cilia, stained with acetylated tubulin (Iomini et al. 2004). Therefore, the difference in the ciliation status of HUVEC cultures can be attributed to the difference in the culture conditions used.

For our experiments, we cultured HUVEC of passage 4 in 2 % FBS until confluence and induced ciliation by serum-starvation for 24 hours. We detected less than 1 % ARL13B-positive cilia, by double immunofluorescence against acetylated tubulin and ARL13B (Supplementary Figure 5 A). This result

is consistent with previous report showing low ciliation in confluent HUVEC cultures in low-serum medium (Lim et al. 2015).

Pg. 17. The authors state that NCI-H295R cells do not have cilia or express Arl13b. It seems more accurate to state they do not have Arl13b-positive cilia, as Arl13b gene expression was not directly examined.

RESPONSE: We thank the reviewer for this correction. This is now edited accordingly in the manuscript (page 19 / line 39-40 and page 20 / line 3).

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

Summary

In their manuscript, "Range of SHH Signaling in Adrenal Gland is limited by membrane Contact and Presence of primary cilia", Mateska et al. seek to identify the role of SHH secretion in adrenal gland cells and cancer cell lines. In their model of SHH secretion they find that secreted SHH is bound to lipoproteins, but this form of SHH is inhibited by co-secreted factors, preventing long-range signaling. Therefore, only short range SHH signaling using membrane-bound ligand remains intact. In the receiving cell, this signaling requires a cilium, which the authors also show must stain positive for the cilia protein Arl13b. The issue of how SHH is secreted and travels to target cells is a longstanding one and this work contributes to identifying several relevant mechanisms. Identifying these mechanisms in the adrenal gland thus moves the field forward by defining them in relevant contexts. Furthermore, the authors show that adrenocortical carcinoma cells work around these mechanisms altogether which may be critical in understanding how to target activation of Shh targets in cancer. Overall, the data are well-controlled and appropriately interpreted but there are several remaining issues:

RESPONSE: We thank the reviewer for the encouraging comments.

Major Concerns

1) The paper meanders from mechanism to mechanism and hard for the reader to follow as it is unclear where it is going. The reader would benefit from a road map at the beginning of the manuscript. As is, the manuscript jumps from exovesicular proteins, to lipoproteins, to cilia, and finally to Arl13b positive cilia. Due to this meandering, the proposed model at the end is a bit murky. While each experiment makes sense and the conclusions drawn are appropriate, the flow between them is hard to follow. Perhaps the authors could take the time to establish the potential models in the introduction before experimentally addressing each one.

RESPONSE: We thank the reviewer for suggesting how to improve the introduction. We have now explained the experimental model at the beginning of each Result section. We hope that these changes improve the paper's flow and clarity (page 13 / line 4-5, page 14 / line 15-18, page 16 / line 16-19, page 17 / line 5-7 and 35-37).

2) The authors propose that Arl13b positive cilia are required for mediating SHH. There are several issues to address regarding this data.

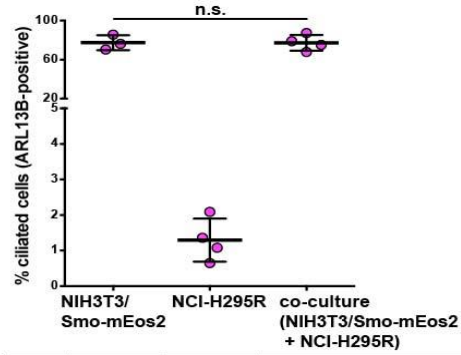
a) If these carcinoma cell phenotypes are a functional result of the cilia being Arl13b negative, Smo and other SHH components would be predicted to be enriched. The authors demonstrate in Figure 6A that these cells express SHH components, including Smo, so this should be examined.

RESPONSE: Shh pathway components are indeed enriched in the cilia of mammalian cells (Bangs and Anderson 2017). However, adrenocortical cells have so few cilia (Figure 7 A – B), that immunofluorescent staining and quantification of Shh pathway components (PTCH1, SMO, GLI2, GLI3) in cilia of NCI-H295R cells would be very difficult. Given that NCI-H295R cells are not responsive to SHH, we decided to not pursue this line of experiments. Furthermore, we do not claim that loss of ARL13B causes the adrenocortical carcinoma cell phenotype. We have never investigated the potential dependency of the adrenocortical carcinoma cell phenotype on the presence of ARL13B in cilia and have no such information from the existing literature.

b) The ciliation of the distinct cell populations in the co-culture is critical and needs clarification. In figure 5, it is unclear how the authors separated NIH3T3-specific cilia from carcinoma cell cilia. The authors have yet to establish the ciliation rates of NIH3T3 and carcinoma lines. Furthermore, Figure 7B shows the carcinoma cells display some cilia so the authors need to explain how NIH3T3 verses carcinoma cell cilia were defined.

RESPONSE: The reviewer is right, we cannot separate NIH3T3-specific cilia from NCI-H295R-specific cilia solely based on immunofluorescence. In the co-culture experiment shown in Figure 5 D - E we stained against ARL13B, a ciliary marker that localizes exclusively in primary cilia. Our data show that only around 1 % of NCI-H295R cells are ARL13B-positive (counted as percentage of ARL13B-positive cells), in contrast to more than 60 % of NIH3T3 cells (Figure 7 B). Therefore, in Figure 5 D - E we considered all ARL13B-positive and SMO-positive cilia to belong to NIH3T3/Smo-mEos2 cells, since their proportion of cilia is vastly greater than the NCI-H295R cells.

To further verify that the ciliation rate of NIH3T3 cells is not affected by the co-culture with carcinoma cells, we cultured NIH3T3/Smo-mEos2 cells alone or in a co-culture with NCI-H295R cells, and immunostained against ARL13B. Quantifying ARL13B-positive cells confirmed that there is no difference in the percentage of ciliated NIH3T3 cells cultured alone or with NCI-H295R cells (Figure 3_for Reviewers only). We added this information on page 16 / line 10.



cells	452	433	592 NIH3T3 + 443 NCI-H295R

Figure 3 for Reviewers only: Quantification of the percentage of ciliated cells in a culture of NIH3T3/Smo-mEos2 cells, NCI-H295R cells or a co-culture of NIH3T3/Smo-mEos2 and NCI-H295R cells, counted as cells positive for ARL13B. The number of counted cells is given under the graph. N ≥ 3 replicates, presented as mean ± SD. Mann-Whitney test.

c) In Figure 7 panel C it is unclear that there are Arl13b negative cilia as the authors state. There are some acetylated α-tubulin positive puncta in the image shown, are there no cilia, or few cilia? Quantification of ciliation in vivo would be informative and support the model proposed in 7D.

RESPONSE: We thank the reviewer for this helpful advice. We have now counted the acetylated tubulin- and ARL13B-positive cilia in the cortex and capsule of adrenal glands and these data are presented in Figure 7 D. The adrenal cortex contains less than 10% ARL13B-positive cells while it contains around 50% acetylated tubulin-positive cells, whereas the adrenal capsule contains 60 – 80 % ARL13B-and acetylated tubulin-positive cells. Hence, the cells of the adrenal capsule are more ciliated than the cells from the adrenal cortex and the adrenocortical cells do contain ARL13B-negative cilia (Figure 7 D, page 17 / line 15-18).

d) Finally, the language describing the role of Arl13b in the SHH pathway should be amended as Arl13b is not a trafficking protein, but like most GTPases it influences the trafficking of membrane proteins. Arl13b is known to influence GliA and not GliR forms, this need correcting in the discussion.

RESPONSE: We thank the reviewer for this correction. We have now corrected the language describing the role of ARL13B in trafficking of Hh pathway components (page 17 / line 12-13 and page 19 / line 33-34).

3) The authors should amend their discussion to include the role of Dispatched in cholesterolated Hh secretion. The Anderson lab previously showed that juxtracrine SHH signaling in the notochord of embryos lacking Dispatched maintains the notochord, which is relevant here as it represents membrane to membrane signaling.

RESPONSE: The role of Dispatched in Shh signaling in the notochord of embryos was added in the discussion (page 19 / line 22-25).

4) In figure 1E, the representative blots are faint and difficult to interpret. In this panel it is not clear that higher density fractions are enriched with SHH as the authors claim. Moreover, this assertion is softened to a partial association with SHH in Figure 3, where the role of exovesicles is dismissed entirely. These inconsistencies are confusing and diminish clarity of the manuscript.

RESPONSE: We provide now blots with a higher exposure in Figure 1 E, which clearly show that the higher density fractions from S150 adrenal supernatant are enriched with SHH, in contrast to the higher density fractions from S16 adrenal supernatant. Upon additional revision of the co-localization and co-immunoprecipitation experiments of SHH with exovesicles (Figure 1 C – E and Figure 3 B – D in the previous manuscript) and as suggested by Reviewer 1, we now limit our observations to association of secreted SHH with lipoprotein particles (APOA1, APOE) (Figure 1 C – E, page 13 / line 11-13 and Figure 3 C – D, page 14 / line 5-9).

Minor Concerns

There are a few places where the description of the result precedes the explanation of the experiment. One example- on page 11 paragraph 2, the second sentence beginning with "We observed..." needs to come after the subsequent explanation of what was done. A proofreading for this will easily fix it.

RESPONSE: We have now changed these instances throughout the manuscript.

The authors should clarify the differences between cell lines used in figure 5. The Shh-LIGHT2 cells are NIH3T3, but distinct from those used in co-cultured experiments. An explanation of why the Shh-LIGHT2 cells were not used for these co-culture experiments would also clarify the experimental details.

RESPONSE: We thank the reviewer for pointing this out. A detailed description of each cell line is now provided in the Materials and Methods section (page 5), as well in the legend of Figure 5. Both Shh-LIGHT2 and NIH3T3/Smo-mEos2 reporter cell lines are NIH3T3 fibroblasts, used for assaying Shh pathway activation at different levels of the signaling cascade. The Shh-LIGHT2 cells express a *firefly luciferase* under the control of a Gli1-dependent promoter, allowing us to quantify Shh pathway – dependent transcription. The NIH3T3/Smo-mEos2 cells are engineered with a construct of SMO fused to the fluorescent protein mEos2, allowing us to follow SMO localization by immunofluorescence.

There is not a clear indicator of how many cells and cilia were examined in experiments shown in figures 5 and 7.

RESPONSE: This missing information is now added in the respective figures.

The font of the graphs of several figures is too small and difficult to read. The x-axis labeling of Figure 5C is confusing. SAG treated control cells should be moved closer to the untreated controls.

RESPONSE: Both points are now amended. Please note that the graph shown in 5 C in the previous manuscript is now shifted to panel 5 E.

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August 25, 2020

RE: JCB Manuscript #201910087R

Dr. Ivona Mateska
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Dresden 01307
Germany

Dear Dr. Mateska,

Thank you for submitting your revised manuscript entitled "Range of Shh signaling in adrenal gland is limited by membrane contact and presence of primary cilia". You will see that they are supportive of further consideration but raise final issues that necessitate your attention. Further experimentation should not be needed but final revisions are required to improve and clarify the data presentation and discussion. While we would not require data to address Reviewer #1's remaining points, please provide a response. It will be important to clarify your statements and resolve the confusion pointed out by Reviewer #1. Please also address Reviewers #2-3's points in the text of the manuscript, including revisions to reconcile the data with current models of Shh response in relation to Arl13b and discussion of the results in the appropriate scholarly context. In addition, the association of Shh with lipoproteins is rather weak (based on the faint WB signals for Shh in Fig. 3D). The association of SHH with lipoproteins rests on an abundant body of literature but we feel that the manuscript would benefit from a better presentation and discussion of the data. For instance, are there other other types of Lpp besides APOA1 and APOE that SHH may associate with in your system?
Overall, we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below) and pending resolution of the points above.

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

1) eTOC summary: A 40-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.
- Please include a summary statement on the title page of the resubmission. It should start with "First author name(s) et al..." to match our preferred style.
Revisions are needed to match our desired style

2) Please be sure to provide tables as stand-alone, editable files (e.g., Word, Excel files, etc.)

3) 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: 5B

4) 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.
- 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.).

5) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication.
- Please abbreviate the names of journals according to PubMed.

6) 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, including supplemental movies and tables if any are included.

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Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Maxence Nachury, PhD
Monitoring Editor, Journal of Cell Biology

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

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

The authors have added new data and significantly revised their manuscript in this revision, which is commendable. However, there are residual major concerns:

1. In their response to my concern that Hedgehog pathway regulation in the adrenal gland is not recapitulated by the carcinoma cell line NCK-H295R, the authors argue that these two are indeed "substantially different", but that they able "resemble" one another. This is confusing, and data from primary adrenocortical cells would greatly help to clear up this issue. Hedgehog transcriptional assays take as little as 12-24 hours to perform in vitro, and thus, the length of time these cells survive in culture (24-48 hours) should be more than sufficient.

2. The new biochemical fractionation data in Figure 4 are a fantastic addition, and the discovery of endocannabinoid-like molecules that may inhibit the HH pathway is intriguing. Does N-acyldopamine 20:4 compete with either BODIPY-Cya or SAG for SMO binding and activation? Further pharmacologic validation of this novel Hedgehog pathway inhibitor would strength the authors' conclusions.

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

In this revised manuscript, Mateska et al. provide additional analysis of Shh signaling in the normal adrenal gland and in adrenocortical carcinoma cells. Overall, the paper is notably improved as a

result of: more thorough analysis of ciliogenesis in adrenal cortex and NCI-H295R cells; further investigation of the properties of the signaling-inhibitory molecule released by NCI-H295R cells, including the possibility that endocannabinoids are responsible; demonstration that normal adrenal gland homogenate and primary adrenal cell culture supernatant also inhibit Shh signaling in NIH-3T3 cells; and improved flow and organization of the manuscript. Thus my concerns are largely addressed, and I believe the paper would be suitable for publication in JCB provided two final issues are addressed (see below).

1. The finding that adrenal gland homogenate and primary adrenal cell culture supernatant also inhibit the Hedgehog pathway is important. However, can the authors comment further on the amount of adrenal gland homogenate or primary adrenal cell culture supernatant used in Fig. 4F-G? The inhibitory effects are only convincing at the highest doses applied, but it is unclear how these doses compare to physiologic concentrations or to the amounts used in experiments with NCI-H295R cells. Such information would help the reader assess the relative potency of inhibition across these samples (if, for example, normalized by amount of Shh present).

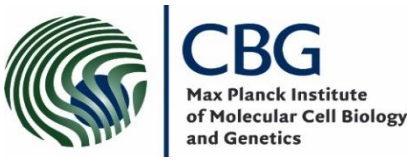
2. It seems a bit premature to concludes that N-acyldopamine 20:4 is likely to be the inhibitory factor released from NCI-H295R cells based on the limited evidence presented. It is clear that the inhibitory factor examined in this paper acts at the level of Smo (or possibly downstream) and inhibits Smo ciliary trafficking. prior work from this group identified the inhibitory activity of endocannabinoids (PMID 25733905), but in that study endocannabinoids were found to inhibit Shh signaling without diminishing Smo ciliary accumulation. Thus, there is a notable difference in the effect of endocannabinoids versus adrenal lipoproteins on Smo ciliary trafficking, which suggests endocannabinoids are not the sole or primary inhibitory factor released by adrenocortical cells. This point should be directly acknowledged in the manuscript, and the suggestion that endocannabinoids are responsible for the inhibitory activity of adrenal cell lipoproteins should likely be tempered.

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

In their revised manuscript, Mateska et al. addressed many reviewer comments including clarifying the narrative. These improvements make the manuscript easier to follow and the additional data strengthen the conclusions. The work is important as it addresses a fundamental question regarding how morphogen signaling is controlled.
A critical issue remains for how the proposed model interprets one aspect of the data. The authors conclude that cancer cells are likely unresponsive to Shh because 95% of cells lack cilia. This is well supported by the field and fits with the data shown here. The authors extrapolate this to healthy adrenal cortical cells that are also unresponsive to Shh. However, these cells are decently ciliated (~50%), with only a few of the cilia being Arl13b positive. While an absence of cilia is predicted to result in an absence of Shh response, the conclusion that these cells are unresponsive to Shh because their cilia lack Arl13b is not tested. The lack of Arl13b in these cilia is correlative to the lack of Shh response. Moreover, loss of Arl13b results in low level activation of Shh activity (regardless of stimulation) and loss of Arl13b in cilia results in normal Shh response. Therefore, the proposed model does not fit with the current understanding of Shh response in relation to Arl13b and needs to be modified.
An additional issue concerns the wording describing the ciliation rates. One example: in Figure 7 it is implied that 50% of cilia are Actub positive and Arl13b negative, whereas 10% of cilia are Actub negative and Arl13b positive. More than likely, what the authors mean to state is 50% of cells are

Actub positive, and of those ciliated cells only 10% are Arl13b positive. Such clarifications will improve reader comprehension.

2nd Revision - Authors' Response to Reviewers: September 1, 2020



Ivona Mateska, PhD
Pfotenhauerstrasse 108
01307 Dresden, Germany
0049 351 210-2633
mateska@mpi-cbg.de

Dresden, September 01, 2020

Dear Dr. Casadio, dear Dr. Nachury,

We are very happy with the positive assessment of our revised manuscript and thank you and the reviewers for the additional comments. We addressed the remaining issues raised and formatted the manuscript according to the JCB guidelines. Additionally, to better address the concern of Reviewer #3, we changed the wording of the title to “Range of Shh signaling in adrenal gland is limited by membrane contact to cells with primary cilia”
Please find appended our response to the reviewers. Changes in the manuscript are highlighted in yellow.

Thank you for submitting your revised manuscript entitled "Range of Shh signaling in adrenal gland is limited by membrane contact and presence of primary cilia". You will see that they are supportive of further consideration but raise final issues that necessitate your attention. Further experimentation should not be needed but final revisions are required to improve and clarify the data presentation and discussion.
While we would not require data to address Reviewer #1's remaining points, please provide a response. It will be important to clarify your statements and resolve the confusion pointed out by Reviewer #1. Please also address Reviewers #2-3's points in the text of the manuscript, including revisions to reconcile the data with current models of Shh response in relation to Arl13b and discussion of the results in the appropriate scholarly context.
- Please see below the responses to the Reviewers.

In addition, the association of Shh with lipoproteins is rather weak (based on the faint WB signals for Shh in Fig. 3D). The association of SHH with lipoproteins rests on an abundant body of literature but we feel that the manuscript would benefit from a better presentation and discussion of the data. For instance, are there other types of Lpp besides APOA1 and APOE that SHH may associate with in your system?
- Based on the existing literature (Palm et al., 2013) and the co-fractionation of SHH from adrenal gland tissue and adrenocortical carcinoma cells with low-density lipoproteins (Figure 1 – 3), we considered the possibility that APOB may associate with SHH. We immunoprecipitated APOB from NCI-H295R-derived conditioned medium and saw that SHH co-immunoprecipitates in the Eluate fraction (data not shown). However, using several antibodies to APOB, we could not convincingly

demonstrate a specific immunoprecipitation of the APOB protein. Therefore, we do not include these data in the manuscript. We acknowledge in the discussion that other lipoproteins or different vehicles may also associate with SHH secreted from adrenocortical cells (page 16 / line 32-35).

Overall, we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below) and pending resolution of the points above.

1) eTOC summary: A 40-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.
- Please include a summary statement on the title page of the resubmission. It should start with "First author name(s) et al..." to match our preferred style.
****Revisions are needed to match our desired style****
- We have now provided an eTOC summary on the title page, matching the desired JCB style.

2) Please be sure to provide tables as stand-alone, editable files (e.g., Word, Excel files, etc.)

3) 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: 5B
- The number of independent data points is now indicated in each figure legend. In Figure 5 B, the data are from n = 12 – 24 replicates, pooled from 3 – 6 independent experiments.

4) 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.
- Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
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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.).

- We added a detailed description of the required information about the microscope image acquisition in the Materials and Methods section “Image acquisition and analysis”.

5) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication.
- Please abbreviate the names of journals according to PubMed.

- The names of the journals used as references are now abbreviated accordingly.

6) 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, including supplemental movies and tables if any are included.

- Description of the online supplementary material is now added at the end of the Materials and Methods section.

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

The authors have added new data and significantly revised their manuscript in this revision, which is commendable. However, there are residual major concerns:

1. In their response to my concern that Hedgehog pathway regulation in the adrenal gland is not recapitulated by the carcinoma cell line NCI-H295R, the authors argue that these two are indeed "substantially different", but that they able "resemble" one another. This is confusing, and data from primary adrenocortical cells would greatly help to clear up this issue. Hedgehog transcriptional assays take as little as 12-24 hours to perform in vitro, and thus, the length of time these cells survive in culture (24-48 hours) should be more than sufficient.

- In our study we address two aspects of the Shh signaling in adrenocortical cells: secretion of the SHH ligand and Shh pathway activity. We find that both healthy and cancerous adrenocortical cells can secrete SHH in association with lipoproteins, but this form of SHH does not activate the Shh pathway. In contrast, the Shh pathway is active only in adrenocortical carcinoma cells in response to TGF-β.

	Healthy adrenal glands	**NCI-H295R adrenocortical carcinoma cells**
Secretion of SHH	**SHH secretion and association with lipoproteins** (SHH co-fractionates with APOA1 and APOE	**SHH secretion and association with lipoproteins** (SHH co-immunoprecipitates with APOA1

	in density gradients)	and APOE)
Activity of the Shh pathway	**No expression** of SHH target genes in steroidogenic adrenocortical cells	**Constitutive ectopic expression** of SHH target genes (GLI1 and PTCH1) No response to autocrine canonical or non-canonical SHH signal, but response to TGF-β

2. The new biochemical fractionation data in Figure 4 are a fantastic addition, and the discovery of endocannabinoid-like molecules that may inhibit the HH pathway is intriguing. Does N-acyldopamine 20:4 compete with either BODIPY-Cya or SAG for SMO binding and activation? Further pharmacologic validation of this novel Hedgehog pathway inhibitor would strength the authors' conclusions.

- The reviewer #1 is proposing interesting experiments to further validate the Shh pathway inhibitor. However, the setup of these experiments and the generation of the required data is not feasible within the given time (7 days) for the final revision of the paper.

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

In this revised manuscript, Mateska et al. provide additional analysis of Shh signaling in the normal adrenal gland and in adrenocortical carcinoma cells. Overall, the paper is notably improved as a result of: more thorough analysis of ciliogenesis in adrenal cortex and NCI-H295R cells; further investigation of the properties of the signaling-inhibitory molecule released by NCI-H295R cells, including the possibility that endocannabinoids are responsible; demonstration that normal adrenal gland homogenate and primary adrenal cell culture supernatant also inhibit Shh signaling in NIH-3T3 cells; and improved flow and organization of the manuscript. Thus, my concerns are largely addressed, and I believe the paper would be suitable for publication in JCB provided two final issues are addressed (see below).

1. The finding that adrenal gland homogenate and primary adrenal cell culture supernatant also inhibit the Hedgehog pathway is important. However, can the authors comment further on the amount of adrenal gland homogenate or primary adrenal cell culture supernatant used in Fig. 4F-G? The inhibitory effects are only convincing at the highest doses applied, but it is unclear how these doses compare to physiologic concentrations or to the amounts used in experiments with NCI-H295R cells. Such information would help the reader assess the relative potency of inhibition across these samples (if, for example, normalized by amount of Shh present).

- We tested at least three volumes of primary adrenal cell culture supernatant or adrenal gland homogenate, differing by one order of magnitude (0.1 µl, 1 µl, 10 µl). In both cases, the lowest volume

required for Shh pathway inhibition was 1 μl (Figure 4 F, G), which corresponds to 1 % from the primary adrenal cell culture supernatant (cells from one adrenal gland were cultured in 100 μl) or 2 % from the adrenal gland homogenate (one adrenal gland was lysed in 50 μl PBS). This corresponds to the secretome of ~ 500 adrenal cultured cells (we obtained ~ 50,000 cells in culture per single adrenal gland). In contrast, the lowest amount of NCI-H295R-supernatant showing Shh pathway inhibitory activity (Figure 4 D, E, H) corresponds to the secretome of ~ 55,000 NCI-H295R cells, implying that the primary adrenal cell culture supernatant has at least 100 x more potent inhibitory effect than the NCI-H295R-supernatant. Hence, the healthy adrenal gland cells release higher amounts or more potent Shh pathway inhibitor(s) than the NCI-H295R adrenocortical carcinoma cells. We added this information in the manuscript (page 13 / line 6-11).

2. It seems a bit premature to conclude that N-acyldopamine 20:4 is likely to be the inhibitory factor released from NCI-H295R cells based on the limited evidence presented. It is clear that the inhibitory factor examined in this paper acts at the level of Smo (or possibly downstream) and inhibits Smo ciliary trafficking. Prior work from this group identified the inhibitory activity of endocannabinoids (PMID 25733905), but in that study endocannabinoids were found to inhibit Shh signaling without diminishing Smo ciliary accumulation. Thus, there is a notable difference in the effect of endocannabinoids versus adrenal lipoproteins on Smo ciliary trafficking, which suggests endocannabinoids are not the sole or primary inhibitory factor released by adrenocortical cells. This point should be directly acknowledged in the manuscript, and the suggestion that endocannabinoids are responsible for the inhibitory activity of adrenal cell lipoproteins should likely be tempered.

- We show that the Shh pathway inhibitor acts at the level or downstream of SMO, but we have not examined its effect on SMO ciliary trafficking. Therefore, based on the data we have, we suggest that endocannabinoids from adrenocortical cells, and N-acyldopamine 20:4 in particular, can inhibit Shh signalling, but we do not investigate the implicated molecular mechanisms. We agree, however, that endocannabinoids may not be the sole or primary inhibitory factor(s) released by adrenocortical cells and we acknowledge in the discussion that other molecules can contribute to the inhibitory activity. We tempered our conclusion accordingly (page 13 / line 31, page 17 / line 3-5).

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

In their revised manuscript, Mateska et al. addressed many reviewer comments including clarifying the narrative. These improvements make the manuscript easier to follow and the additional data strengthen the conclusions. The work is important as it addresses a fundamental question regarding how morphogen signaling is controlled.

1. A critical issue remains for how the proposed model interprets one aspect of the data. The authors conclude that cancer cells are likely unresponsive to Shh because 95% of cells lack

cilia. This is well supported by the field and fits with the data shown here. The authors extrapolate this to healthy adrenal cortical cells that are also unresponsive to Shh. However, these cells are decently ciliated (~50%), with only a few of the cilia being Arl13b positive. While an absence of cilia is predicted to result in an absence of Shh response, the conclusion that these cells are unresponsive to Shh because their cilia lack Arl13b is not tested. The lack of Arl13b in these cilia is correlative to the lack of Shh response. Moreover, loss of Arl13b results in low level activation of Shh activity (regardless of stimulation) and loss of Arl13b in cilia results in normal Shh response. Therefore, the proposed model does not fit with the current understanding of Shh response in relation to Arl13b and needs to be modified.

- We thank the reviewer for this comment and agree that our findings do not directly prove that lack of ARL13B-positive cilia mediates the absence of SHH responsiveness in healthy adrenocortical cells. However, our data clearly demonstrate a correlation between the absence of SHH responsiveness and the lack of ARL13B-positive cilia in healthy adrenocortical cells, in contrast to capsule cells, which contain ARL13B and respond to SHH.

In the developing mouse neural tube, loss of Arl13b results in constitutively low levels of Gli activators which cannot be modified to create high-level activators in response to Hh stimulation, but no effect on Gli3 processing to its repressor form (Caspary et al., 2007). In microdissected mouse adrenal cortex lacking ARL13B, we see no *Gli2* expression *and* only a very low *Gli3* expression (Figure 6 A). This suggests that in absence of ARL13B adrenocortical cells do not express Gli2/3 required to induce *Gli1*, which is consistent with the current model of Shh response. It is also shown that ARL13B can function outside of the cilium to regulate Shh signaling (Mariani et al., 2016; Gigante et al., 2020), however, with our immunofluorescence method, we do not detect any ARL13B outside the cilia (Figure 7 A, C). It is possible that adult adrenocortical cells behave similarly to Arl13b-null cells in their Shh response. Additionally, we do not know whether other ciliary proteins might influence the Shh pathway activity in adrenocortical cells.

We have adjusted the interpretation of our data accordingly (page 3 / line 26-30, page 15 / line 23-24 & 33-35 and page 18 / line 10-15).

2. An additional issue concerns the wording describing the ciliation rates. One example: in Figure 7 it is implied that 50% of cilia are Actub positive and Arl13b negative, whereas 10% of cilia are Actub negative and Arl13b positive. More than likely, what the authors mean to state is 50% of cells are Actub positive, and of those ciliated cells only 10% are Arl13b positive. Such clarifications will improve reader comprehension.

- Acetylated tubulin and ARL13B co-localize in the cilia of NIH-3T3 fibroblasts and NCI-H295R cells (Figure 7 A and Supplementary Figure 4). However, we did not perform a double staining of acetylated tubulin and ARL13B in the mouse adrenal gland. Based on our staining, we show that the adrenal capsule contains ~ 80 % acetylated tubulin-positive and ~ 60 % ARL13B-positive cells, while the adrenal cortex contains ~ 50 % acetylated tubulin-positive and less than 10 % ARL13B-positive cells. Hence, the ciliary protein ARL13B is significantly less abundant in the adrenocortical than in the capsule cells (page 15 / line 20-23).