Time-resolved proteomics profiling of the ciliary Hedgehog response

Elena May, Marian Kalocsay, Inès Galtier D'Auriac, Patrick Schuster, Steve Gygi, Maxence Nachury, and David Mick

Corresponding Author(s): David Mick, Saarland University

Review Timeline:

Submission Date:	2020-08-10
Editorial Decision:	2020-09-29
Revision Received:	2021-02-01
Editorial Decision:	2021-02-22
Revision Received:	2021-03-02

Monitoring Editor: Jodi Nunnari

Scientific Editor: Melina Casadio

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

DOI: https://doi.org/10.1083/jcb.202007207

September 30, 2020

Re: JCB manuscript #202007207

Dr. David U Mick
Saarland University
Center of Human and Molecular Biology (ZHMB)
Kirrberger Str. 100
Homburg 66421
Germany

Dear Dr. Mick,

Thank you for submitting your manuscript entitled "Time-resolved proteomic profiling of the ciliary Hedgehog response reveals that GPR161 and PKA undergo regulated co-exit from cilia". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We sincerely apologize for the delay in sending you this decision. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that both reviewers appreciated the quality of the proteomics datasets and found them of high interest. Reviewer #1 suggested strengthening the new observations derived from the proteomics analyses, which in our view would be a compelling way to show the value of the datasets to generate new biological insight about ciliary signaling. We strongly encourage you to consider their points seriously. We would be happy to further discuss the revisions if you anticipate any issues addressing the reviewer remaks or have any questions.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for Tools 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: Tools may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://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.

Tools 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.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

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 in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief, Journal of Cell Biology

Melina Casadio, Ph.D.
Senior Scientific Editor, Journal of Cell Biology

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

The manuscript by May et al describes improvements in the cilia-APEX2 proximity labeling scheme using state-of-the-art quantitative mass spectrometry with tandem-mass-tags. Unbiased description of the mammalian primary cilia proteome has remained challenging, as mammalian cilia cannot be readily isolated without impurities. The described method allows identification of several ciliary signaling proteins of high importance and low abundance, such as Ptch1, Gpr161 and Smo that were not detected in previous APEX-based methods. By utilizing several controls, careful selection of criteria and including additional CLIME analysis, the authors provide time-resolved changes in ciliary proteome with high confidence. This approach correctly identifies the new factors that undergo Hedgehog-regulated ciliary redistribution including PKA regulatory subunit R1alpha and Paladin. The authors also propose a revised model regarding Gpr161 removal from cilia with relation to their interaction with PKA regulatory subunits. The manuscript is well written, and the described methods are timely in their broad implications in understanding of dynamic subcellular constituents of minute subcellular compartments such as cilia.

I have a few comments that might strengthen the proposed features of the model in Fig. 5 describing removal of Gpr161 from cilia.

1. The authors nicely show that lack of PKARIalpha results in retention of Gpr161 in cilia. They suggest that PKARIalpha/Gpr161 is dissociated from PKA-c at resting concentrations of cAMP in cilia and that Smo or Sstr3 activation causes reduction of cAMP levels promoting binding of PKA-c with PKA-R1/Gpr161. However, PKA-c has so far been reported to localize at the basal body. If this early phase of recruitment of PKA-c to Gpr161/PKA-RIalpha in ciliary membrane can be demonstrated, it will unambiguously establish ciliary distribution of PKA-c according to the authors' model.

2. The recruitment of beta-arrestin to Gpr161 requires a C-terminal region proximal (Pal et al 2016) to the proposed PKA phosphorylation site mentioned in Bachmann et al., 2016. The authors propose that PKA-c recruitment to C-tail results in phosphorylation of the C-tail and increases beta-arrestin recruitment. Grk-2/3 also has been proposed to phosphorylate Gpr161 C-tail in beta-arrestin recruitment (Pal et al. 2016). If the authors can demonstrate that β-arrestin recruitment to Gpr161 is altered by mutants of the proposed PKA-c site, it will unambiguously establish their proposed model.

Minor points.

1. Please show individual Gli3 full-length and Gli3R data (rather than only showing ratios) in Fig. 8-B/C.

2. Line 374: Typo GPCR.

3. Lines 430-32: Please add references.

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

This manuscript by May and co-workers uses proximity biotinylation to characterize the ciliary proteome. To date, adequate methods of purifying primary cilia for proteomics are lacking and proximity biotinylation provides a workaround. This paper builds on prior work from the group, which used an older generation of Apex for labeling. In the current work, they use Apex2, which is an Apex variant with higher turnover. This allowed for lower levels of Nphp3-Apex2 in the cilia, which perturbed the cilia less. Apex2 also allowed for faster labeling, making it possible to look at changes in ciliary composition minutes after the activation of hedgehog signaling. This allowed detailed analysis of the kinetics of movements of hedgehog signaling components and the relationship to cAMP signaling. In addition, they identified PALD1, a phosphatase (or pseudo phosphatase) that localizes to cilia with kinetics like Smo.

The datasets generated by these studies will be of value to the community of researchers studying hedgehog signaling and primary cilia, and will be important to the human genetics community. It is clear that this dataset has significantly fewer false negatives than the previous dataset. For example, only about half of the IFT subunits and no BBSome subunits were found in the previous dataset but these are now covered with only a few exceptions. It is hard to judge false positives but I am optimistic that the relatively small size of the dataset will make the ratio of true to false positives favorable.

Other Points:

1) The manuscripts lacks of measurements of effects of PALD1 on gene expression in the knockout IMCD3, 3T3 and C2C12 cells. The authors showed extensive data on the redistribution of components, but never actually show how this affects the readout of the pathway by measuring Gli1 or Ptch1 expression. This omission makes it hard to understand how important this protein is to hedgehog signaling.

2) Legends describing the tables should be strengthened. The relationships between the datasets are not evident and it is not straightforward to understand what is shown in the spreadsheets.

3) Figure 4C: Should the x axis be minutes rather than hours?

4) Is Pkd1 moved into cilia in response to hedgehog? This is an interesting observation but not confirmed.

5) Page 9: something is wrong with this reference (Taylor et al., 2012, 201).

Saarland University School of Medicine
66421 Homburg | Germany

The Journal of Cell Biology
The Rockefeller University Press
950 Third Ave, 2nd floor
New York, NY 10022
USA

Jun.-Prof. Dr. David U. Mick
Saarland University School of Medicine
Center of Human and Molecular Biology (ZHMB)
Campus Homburg, Bldg. 45.2
66421 Homburg/Saar

T: +49 (0) 6841 16-26577
F: +49 (0) 6841 16-26587

Homburg & San Francisco, 1 Feb. 2021

Subject: Resubmission of revised JCB manuscript# 202007207 - May *et al.*

Dear Jodi,

We would like to thank the expert reviewers for their valuable comments on our manuscript “Proteomic profiling of the ciliary Hedgehog response reveals coordinated exit of GPR161 and PKA”. We very much appreciate the reviewers’ and your efforts to help us strengthen our manuscript, especially to highlight the value of our proteomic analyses to generate new biological insights into ciliary signaling.

We also appreciate JCB’s policy to waive the revision time limit, which allowed us to attempt to experimentally address all points raised by the reviewers despite limited operations in our institutions.

We have attached a point-by-point response to all reviewers’ comments below (in blue) and are happy to submit a revised manuscript with new experimental data.

We have significantly edited the manuscript for clarity and brevity. The main text is now 33.328 characters, and the legends are 11.511 characters.

We hope that you and the reviewers are satisfied with our revisions and consider the manuscript appropriate for publication in the Journal of Cell Biology.

Best regards,

David Mick & Maxence Nachury

<u>Reviewer #1</u> (Comments to the Authors (Required)):

The manuscript by May et al describes improvements in the cilia-APEX2 proximity labeling scheme using state-of-the-art quantitative mass spectrometry with tandem-mass-tags. Unbiased description of the mammalian primary cilia proteome has remained challenging, as mammalian cilia cannot be readily isolated without impurities. The described method allows identification of several ciliary signaling proteins of high importance and low abundance, such as Ptch1, Gpr161 and Smo that were not detected in previous APEX-based methods. By utilizing several controls, careful selection of criteria and including additional CLIME analysis, the authors provide time-resolved changes in ciliary proteome with high confidence. This approach correctly identifies the new factors that undergo Hedgehog-regulated ciliary redistribution including PKA regulatory subunit R1alpha and Paladin. The authors also propose a revised model regarding Gpr161 removal from cilia with relation to their interaction with PKA regulatory subunits. The manuscript is well written, and the described methods are timely in their broad implications in understanding of dynamic subcellular constituents of minute subcellular compartments such as cilia.

I have a few comments that might strengthen the proposed features of the model in Fig. 5 describing removal of Gpr161 from cilia.

1. The authors nicely show that lack of PKARIalpha results in retention of Gpr161 in cilia. They suggest that PKARIalpha/Gpr161 is dissociated from PKA-c at resting concentrations of cAMP in cilia and that Smo or Sstr3 activation causes reduction of cAMP levels promoting binding of PKA-c with PKA-R1/Gpr161. However, PKA-c has so far been reported to localize at the basal body. If this early phase of recruitment of PKA-c to Gpr161/PKA-RIalpha in ciliary membrane can be demonstrated, it will unambiguously establish ciliary distribution of PKA-c according to the authors' model.

We are grateful for the reviewer's input to help strengthen our proposed model. During the revision, we attempted to test whether PKA-C accumulates in primary cilia during the course of the Hedgehog response via multiple independent approaches (immunofluorescence microscopy, live imaging of cell lines stably expressing fluorescently-tagged PKA-C, transient transfections into multiple cell lines). Despite our best efforts, these experiments remain difficult to interpret. In a nutshell, the diffuse cytoplasmic signals of PKA-C obscure any signal that may be present in cilia. The imaging analyses are consistent with the mass-spectrometric data, which detect considerable non-ciliary (most likely cytosolic) signals of PKA-C. Hence, we clarified in the text (line 288-290) that our model is lacking definitive proof of PKA-C localization to

cilia under physiological conditions, while maintaining our hypothetical model as it best describes all observations (see next point raised by the reviewer).

2. The recruitment of beta-arrestin to Gpr161 requires a C-terminal region proximal (Pal et al 2016) to the proposed PKA phosphorylation site mentioned in Bachmann et al., 2016. The authors propose that PKA-c recruitment to C-tail results in phosphorylation of the C-tail and increases beta-arrestin recruitment. Grk-2/3 also has been proposed to phosphorylate Gpr161 C-tail in beta-arrestin recruitment (Pal et al. 2016). If the authors can demonstrate that β-arrestin recruitment to Gpr161 is altered by mutants of the proposed PKA-c site, it will unambiguously establish their proposed model.

To address the reviewer's point, we mutated the putative PKA phosphorylation sites in the GPR161 C-terminal tail and generated a PKA phospho-mimetic $GPR161_{S445D,S446D}$ and a non-phosphorylatable $GPR161_{S445A,S446A}$ mutant. We assessed the recruitment of β-arrestin2 to both mutants by bioluminescence resonance energy transfer (BRET) assays (new Figure 5H). While the phospho-mimetic mutant shows wild type-like recruitment of β-arrestin2, the phospho-dead mutant shows a marked reduction in β-arrestin recruitment. Considering the findings of Bachmann et al., 2016 that human GPR161 with the same phospho-mimetic mutations shows a marked reduction in cilia accumulation, while the phospho-dead mutant localizes to cilia as well as wild-type, the results of our BRET assays further support our model of PKA-dependent removal of GPR161 from primary cilia.

Minor points.

1. Please show individual Gli3 full-length and Gli3R data (rather than only showing ratios) in Fig. 8-B/C.

Quantitation of GLI3 full-length and GLI3R signals are now provided in the new Figures S4B and S4C.

2. Line 374: Typo GPCR.
Typographical error has been fixed.

3. Lines 430-32: Please add references.
References have been added.

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

This manuscript by May and co-workers uses proximity biotinylation to characterize the ciliary proteome. To date, adequate methods of purifying primary cilia for proteomics are lacking and proximity biotinylation provides a workaround. This paper builds on prior work from the group, which used an older generation of Apex for labeling. In the current work, they use Apex2, which is an Apex variant with higher turnover. This allowed for lower levels of Nphp3-Apex2 in the cilia, which perturbed the cilia less. Apex2 also allowed for faster labeling, making it possible to look at changes in ciliary composition minutes after the activation of hedgehog signaling. This allowed detailed analysis of the kinetics of movements of hedgehog signaling components and the relationship to cAMP signaling. In addition, they identified PALD1, a phosphatase (or pseudo phosphatase) that localizes to cilia with kinetics like Smo.

The datasets generated by these studies will be of value to the community of researchers studying hedgehog signaling and primary cilia, and will be important to the human genetics community. It is clear that this dataset has significantly fewer false negatives than the previous dataset. For example, only about half of the IFT subunits and no BBSome subunits were found in the previous dataset but these are now covered with only a few exceptions. It is hard to judge false positives but I am optimistic that the relatively small size of the dataset will make the ratio of true to false positives favorable.

Other Points:

1) The manuscripts lacks of measurements of effects of PALD1 on gene expression in the knockout IMCD3, 3T3 and C2C12 cells. The authors showed extensive data on the redistribution of components, but never actually show how this affects the readout of the pathway by measuring Gli1 or Ptch1 expression. This omission makes it hard to understand how important this protein is to hedgehog signaling.

We have succeeded in generating additional *Pald1*$^{-/-}$ 3T3 cell lines by genome editing (see new Figure S4E) and analyzed *Gli1* mRNA expression in IMCD3 and 3T3 wild-type and *Pald1*$^{-/-}$ in response to Hedgehog signal, which is presented in the new Figure S4D. The *Gli1* mRNA levels are in agreement with the GLI3 processing data presented in Figure 8C (and new Figures S4B-C), as we observe increased *Gli1* mRNA expression in the absence of PALD1 in unstimulated IMCD3 cells, which can be further stimulated by Shh addition. Interestingly, we only observe minor changes in 3T3 cells upon *Pald1* knockout, consistent with the absence of PALD1 recruitment to cilia in 3T3 cells (see Fig. 5G) indicating a cell-type specific function of PALD1.

2) Legends describing the tables should be strengthened. The relationships between the datasets are not evident and it is not straightforward to understand what is shown in the spreadsheets.

We are grateful for this helpful comment and have reworked both the illustrations of the experimental workflows (Figures 2A, 3A and S3) as well as the table legends to contain more specific information on the presented data, especially in Table S2. We have strengthened the legends to convey the complex data analysis in a more straightforward fashion.

3) Figure 4C: Should the x axis be minutes rather than hours?

We are thankful for the reviewer to noticing the mislabeling of the axis. It has been corrected.

4) Is Pkd1 moved into cilia in response to hedgehog? This is an interesting observation but not confirmed.

Although PKD1 is displayed next to the Smo/Pald1 minicluster in our hierarchical cluster analysis of the proteomic changes upon SHH signal induction, it is quite distant and closer to the non-changing components, as the slight changes are only observed in “set #1”, not “set #2” (see Figure 5A). We do agree that it is an interesting observation, however, due to this discrepancy between the two datasets, we decided not to follow up this observation.

5) Page 9: something is wrong with this reference (Taylor et al., 2012, 201).

We have corrected the erroneous reference.

February 22, 2021

RE: JCB Manuscript #202007207R

Dr. David U Mick
Saarland University
Center of Human and Molecular Biology (ZHMB)
Kirrberger Str. 100
Homburg 66421
Germany

Dear Dr. Mick,

Thank you for submitting your revised manuscript entitled "Proteomic profiling of the ciliary Hedgehog response reveals coordinated exit of GPR161 and PKA". Both reviewers now recommend publication and one referee suggests incorporating new work into the discussion, which we agree is important. We would be happy to publish your paper in JCB pending changes to address Reviewer #1's comments and final revisions necessary to meet our formatting guidelines (see details below).

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

1) JCB Tools must have separate "Results" and "Discussion" sections. Please be sure to format the paper according to our guidelines and make this change prior to resubmission. https://rupress.org/jcb/pages/submission-guidelines#manuscript-prep

2) Titles, eTOC: Please consider the following revision suggestions aimed at increasing the accessibility of the work for a broad audience and non-experts.

Title: Time-resolved proteomics profiling of the ciliary Hedgehog response

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.

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.

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.

- For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features *even if described in other published work or gifted to you by other investigators*
- Please include species and source for all antibodies, including secondary, as well as catalog numbers/vendor identifiers if available.
- Sequences should be provided for all oligos: primers, si/shRNA, gRNAs, etc.
- 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. Abbreviate the names of journals according to PubMed.
- Please check our preprint citation policies for in-text and reference list formatting and reformat as needed:
https://rupress.org/jcb/pages/reference-guidelines
This citation needs to be revised:
"Arveseth, C.D., J.T. Happ, D.S. Hedeen, J.-F. Zhu, J.L. Capener, D.K. Shaw, I. Deshpande, J. Liang, J. Xu, S.L. Stubben, I.B. Nelson, M.F. Walker, N.J. Krogan, D.J. Grunwald, R. Hüttenhain, A. Manglik, and B.R. Myers. 2020. Smoothened Transduces Hedgehog Signals via Activity-Dependent Sequestration of PKA Catalytic Subunits. bioRxiv. 2020.07.01.183079. doi:10.1101/2020.07.01.183079"

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.

7) Tables must be provided in individual, editable files (e.g., Word or Excel). Please make sure the "cell lines" table is numbered and referred to in the text. Tables must be separated from the M&M (or, alternatively, the information needs to be incorporated into the M&M in paragraph form).

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required

prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

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

Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief, Journal of Cell Biology

Melina Casadio, Ph.D.
Senior Scientific Editor, Journal of Cell Biology

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

The authors have satisfactorily responded to all my previous comments. They now also show that the PKA phospho-mimetic GPR161 S445D, S446D mutant has reduced trend (please show statistics) in binding to beta arrestin using BRET assays. This correlates with Bachmann et al 2016 zebra fish data showing lower levels of phospho-mimetic Gpr161 in cilia while non-phosphorylatable Gpr161 in not being unaffected. Please note that a paper in Development (PMID: 33531430) from the same group has now shown that the non-phosphorylatable Gpr161 also shows a significant decrease in ciliary localization in randomly selected cells (unlike previously chosen dorsal midline

cells), and more so compared to wild type upon sonic hedgehog treatment. This suggests that PKA phoshphorylation of Gpr161 is complex in its role in maintaining steady state ciliary levels and in removal from cilia. I would advise that the authors at least discuss this paper and incorporate implications of these new results in light of the proposed model in the Figure 5I.

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

My concerns have been addressed.