Rpgrip1l controls ciliary gating by ensuring the proper amount of Cep290 at the vertebrate transition zone

Antonia Wiegering, Renate Dildrop, Christine Vesque, Hemant Khanna, Sylvie Schneider-Maunoury, and Christoph Gerhardt

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RE: Manuscript #E20-03-0190
TITLE: Rpgrip1l controls ciliary gating by ensuring the proper amount of Cep290 at the vertebrate transition zone

Dear Dr. Gerhardt:

It has taken a while but we were able to get two reviewers for your work, despite the current difficulties. Both reviewers were critical of the manuscript and indicate that they would like to see a considerable number of additional experiments. In particular the use of cell lines needs to be more consistent (reviewer 2) and additional experiments relating to eupatilin and CEP290 function need to be performed (see major points, reviewer 1). Reviewer 1 has some questions about the image quality (reviewer 1, point 1), and I share that view. Can you make sure any revisions fully address this point.

Given the circumstances I don't want to put a time limit on the revisions, which do require considerable additional experimental work.

Sincerely,

Francis Barr
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Gerhardt,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you haveopted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

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In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

Wiegering and colleagues investigated the role of the ciliopathy protein Rpgrip1l in ciliary transition zone formation. In a previous MS (JCB 2015), the same laboratory published that Rpgrip1l controls ciliary signaling by regulating the activity of the proteasome via Psdm2. In this MS, the authors show that Rpgrip1l governs ciliary gating by ensuring the proper amount of Cep290 at the transition zone. Further, they show that the flavonoid eupatilin rescues ciliary gating defects in the absence of Rpgrip1l.

It is already known that Rpgrip1l is required for centrosomal accumulation of Cep290, that Cep290 controls the localization of Arl13b and Ac3 to the cilia and Eupatilin rescues defects in CEP290 KO cells. Here, the novelty is that expression of a FLAG-tagged variant of mouse CEP290 rescues the defects of Rpgrip1l KO cells in promoting Arl13b localization. This leads to the conclusion that Rpgrip1l controls ciliary gating via CEP290. Furthermore, the authors propose that Rpgrip1l functions as a scaffold for Nphp1 but not Cep290 localization at the cilium.

The MS is well written and the topic is very interesting. The data showing that FLAG-mCEP290 rescues the localization of Arl13b in Rpgrip1l KO cells is nice and convincing. However, I am concerned about the quality of images provided, the accuracy of quantifications and some conclusions that are not backed up by proper controls.

Major points:
1. All images are highly contrasted and hardly any background staining is visible. When we analyzed the figures in ImageJ, we noticed the presence of saturated pixels. This needs clarification since saturated images can alter the outcome of signal intensity quantifications.

It is also not clear whether the low background is a result of background subtraction during or after image acquisition or any other type of image editing that the authors did not describe. Please clarify.

It will be important to see the raw data and figures with non-saturated images.

2. I found hard to judge the accuracy of quantifications. It is not clearly stated how many cells/cilia were quantified in total per condition. In all graphs, if each dot represents one cell/cilia, then the number of cells/cilia analyzed was very low. Please provide the relevant information in each figure legend.

3. Scaffolding function of Rpgrip1l:
The authors show that FLAG-mCep290 binds to the TZ in Rpgrip1l KO cells, whereas a construct carrying Myc-NPHP1 does not. Based on these data, they conclude that Rpgrip1l functions as a scaffold for NPHP1 but not for Cep290. I see a couple of problems with this conclusion. First, the FLAG-mCep290 construct might have a dominant effect (due to the tag and/or mouse variant). Hence, it could constantly bind to the TZ independently of Rpgrip1l, yet endogenous Cep290 might still requires Rpgrip1l as a scaffolding protein. Although, this might be difficult to prove, the authors could at least perform one rescue experiment using different tagged version of Cep290 or non-tagged Cep290 (ideally human Cep290) in Rpgrip1l KO cells.

To conclude that Rpgrip1l functions as a scaffold for Nphp1, the authors should show that the Myc-Nphp1 construct is functional and able to bind to the TZ in wild type cells. In figure S3, the authors only show that Myc-Nphp1 does not localize at the TZ in Rpgrip1l null cells. The fact that Myc-Nphp1 is expressed (showed by Western blot), does not mean that it localizes properly.

4. Based on the rescue of Arl13b localization but not cilia length by FLAG-mCep290, the authors conclude that Rpgrip1l regulates different aspects at the TZ. This assumption is plausible, however, the authors should show that FLAG-mCep290 is fully functional in human cells. For ex., it is known that depletion of Cep290 leads to an increase in ciliary length. Can FLAG-mCep290 rescues this phenotype? If not, this would imply the FLAG-mCep290 is only partially functional in human cells.

5. The data related to treatment of Rpgrip1 KO cells using eupatilin is interesting. However, I found the conclusion mis-leading. In page 7, the authors state that "The treatment of Rpgrip1l -/- NIH3T3 cells with eupatilin restored the amount of Arl13b and Sstr3 (Figure 4A and B) indicating that Rpgrip1l controls ciliary gating via ensuring the proper amount of Cep290 at the vertebrate TZ." To be able to conclude this, the authors should show that Eupatilin restores Cep290 levels in Rpgrip1l. Can Eupatilin rescues Arl13b localization in Cep290 KO cells? If it does, the results related to Eupatilin would only show that Eupatilin works downstream of Rpgrip1l and Cep290.

6. In page 10, the authors conclude that "Cspp1 is at the top of the ciliary gating hierarchy involving Rpgrip1l, Cep290 and Nphp5 (Figure 4D)". Could the authors provide experimental evidences for this model? For instance, testing if FLAG-mCep290 and Eupatilin rescue defects of Cspp1 depleted cells. These would be easy to perform experiments that would strengthen the MS.

Minor points:
1. Please provide data showing the specificity of the antibodies used. This is particularly important for results showing no change in protein localization upon gene depletion.

2. Cite papers that previously shown reduction of Arl13b and Ac3 in the absence of Cep290 (e.g. Molinari et al, 2019, Shimada et al., 2017).

3. It is not clear how images were taken (z-stacks? How many? spacing?) and how images were treated for quantifications (quantifications done using sum projections, max. projections?). Please provide detailed information in materials and methods.

4. Figure 3D and E, ciliary length. In the images showed in figure 3D, the cilium of Rpgrip1l -/- cells seems to be approx. 3 times longer than the WT cilium. However, the quantifications in 3E show an average ciliary length increase of only 1.5x in Rpgrip1l -/- cells. Please clarify and show a representative image of the average to avoid mis-conception.

5. It would be more informative if the authors could plot the cilia length measured (in µm) instead of the normalized cilia length in figure 3E.

Reviewer #2 (Remarks to the Author):

In this study, the authors examined the role or RPGRIP1L in ensuring CEP290 proper localization to the TZ to maintain ciliary gating and discuss the therapeutic potential of the flavonoid eupatilin to rescue ciliopathies due to RPGRIP1L mutations. The authors show that Sstr3 is reduced in Rpgrip1l ko cilia. They then go on to show that the ciliary levels of Arl13b and Sstr3 are not affected in NPHP1 and Inversin ko cells, but are reduced in Cep290 inactivated cells.

1. The authors concluded that CEP290 mislocalisation in Rpgrip1l ko cells may underlie the ciliary gating defect. However, CEP290 mislocalisation was not a direct effect of Rpgrip1l loss, as overexpression of FLAG-mCEP290 in Rpgrip1l ko cells rescued the gating defect for Arl13b. This be has the question of how CEP290 is mislocalised in the absence of Rpgrip1l and how such mechanism does not affect the correct localisation of FLAG-mCEP290. Also, what is the reason of using murine and not human CEP290 in these studies, particularly since HEK293 cells are of human origin?

2. The studies started with MEFs but moved on to NIH3T3 cells. Most of the data and cell lines have been published earlier by this group. Does the effect of CEP290 on ciliary Arl13b and Sstr3 also happen in MEFs?

3. The failure of FLAG-mCEP290 transfection in MEFs has not been explained. Earlier studies have shown efficient transfection of CEP290 into these cells. The switch to HEK293 adds another variable to the experiments. The ectopic staining of recombinant NPHP1 should be provided in the same cell where no effect on ARL13B recruitment was observed.

4. The mechanism underlying elongated cilia and lack of effect of CEP290 reconstitution on cilia length has not been dealt with at all in this study. The author's group has also previously shown cilia length extension in Rpgrip1l mutant cells and tissues; however, the mechanism is not provided.

5. The experiment and conclusions of euplatilin treatment seem preliminary at best. The authors discuss a possible mechanism involving NPHP5; however, given the relatively mild phenotype of NPHP5 mutations, like that of NPHP1 and NPHP4, the involvement of NPHP5-mediated mechanisms in Rpgrip1l ciliary gating seems like a stretch.

6. This study is computing in a confusing manner data from MEFs, HEK293 and NIH3T3. If CEP290 overexpression could be a limitation in hard to transfect cells, positive effects of eupatilin treatment should be done in RPGRIIP1L mutant MEFs, in addition to in vivo benefits in the corresponding mice model.

Dear Reviewers,

We appreciate your comments on our manuscript entitled “Rpgrip1l controls ciliary gating by ensuring the proper amount of Cep290 at the vertebrate transition zone” (#E20-03-0190) which gave us invaluable suggestions for the revision of our manuscript. We have taken these comments as a guide for the correction and quality improvement of our manuscript. Every point you made was carefully considered and revised. We have addressed your specific comments in a point-by-point manner. Your questions/comments are written in italic letters, our answers not.

Reviewer #1 (Remarks to the Author):

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It is also not clear whether the low background is a result of background subtraction during or after image acquisition or any other type of image editing that the authors did not describe. Please clarify.

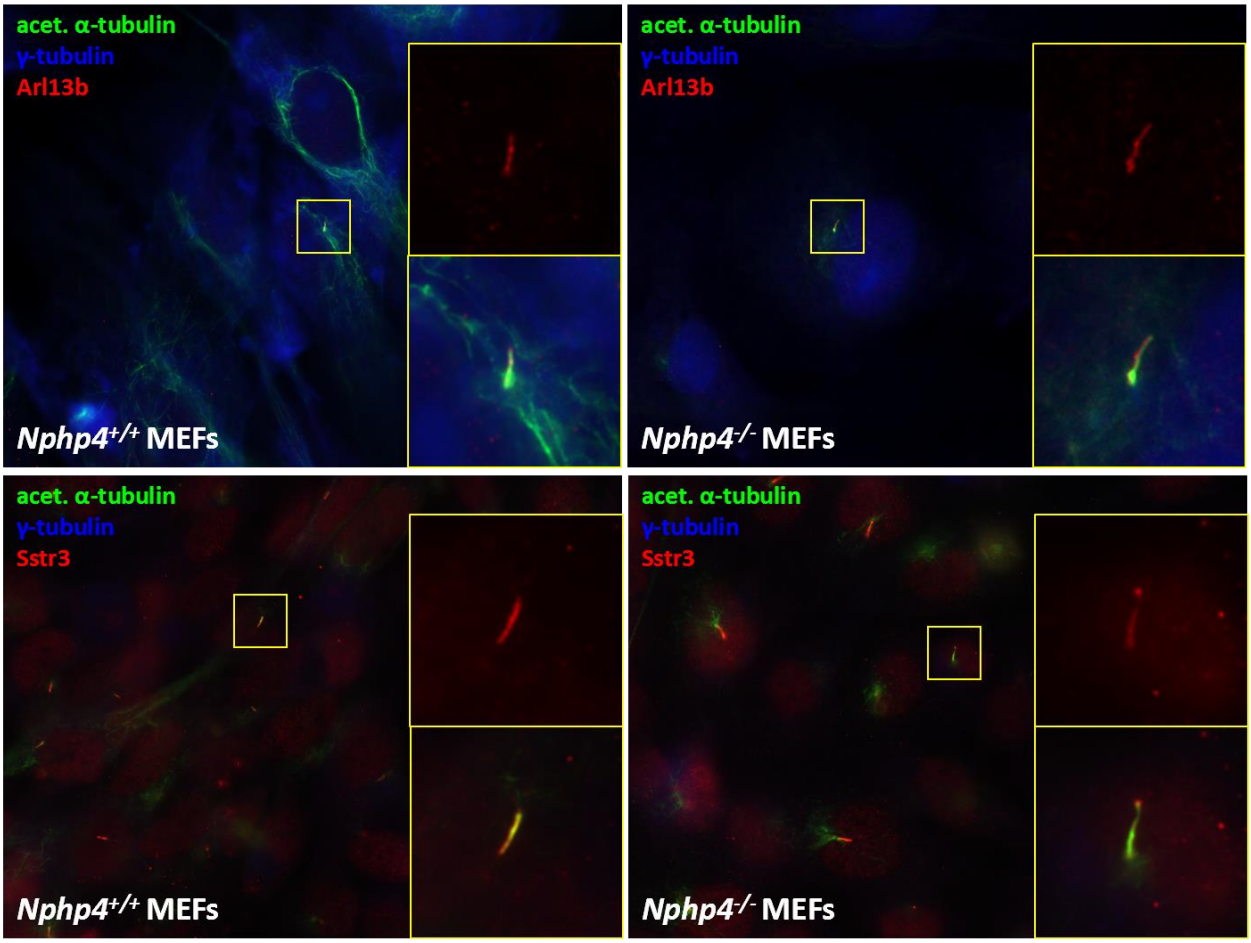
We understand the concerns of Referee #1 regarding the image presentation and corresponding questions of signal intensity quantifications. The quantifications were performed on single plane

raw images without any kind of editing and without measuring oversaturated pixels. In the presented figures, we used optimised images to illustrate our quantification results. Here, we substracted the background and adjusted contrast settings for optimal visualisation. To prevent any misconceptions of that, we replaced the images in all figures of our revised manuscript and refined the respective Material and Method section [see Material and Method Section Image Processing (p. 23), Image Presentation (p. 23) and Quantification (pp.24-25)].

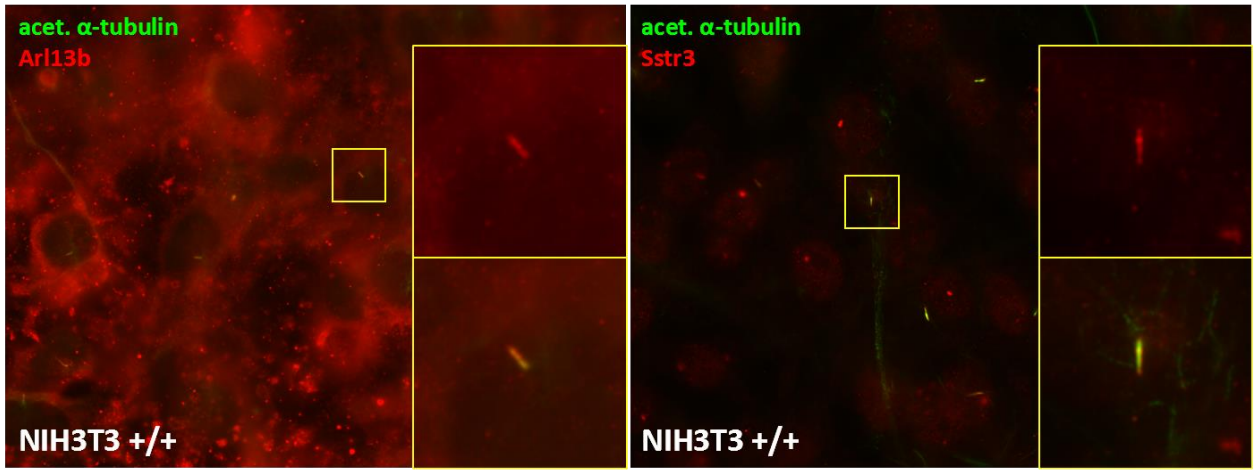
It will be important to see the raw data and figures with non-saturated images.

As mentioned above, we replaced all images in the revised version of our manuscript. We performed our ciliary IF very carefully and hope that the selection of raw images below can dispel your reservations.

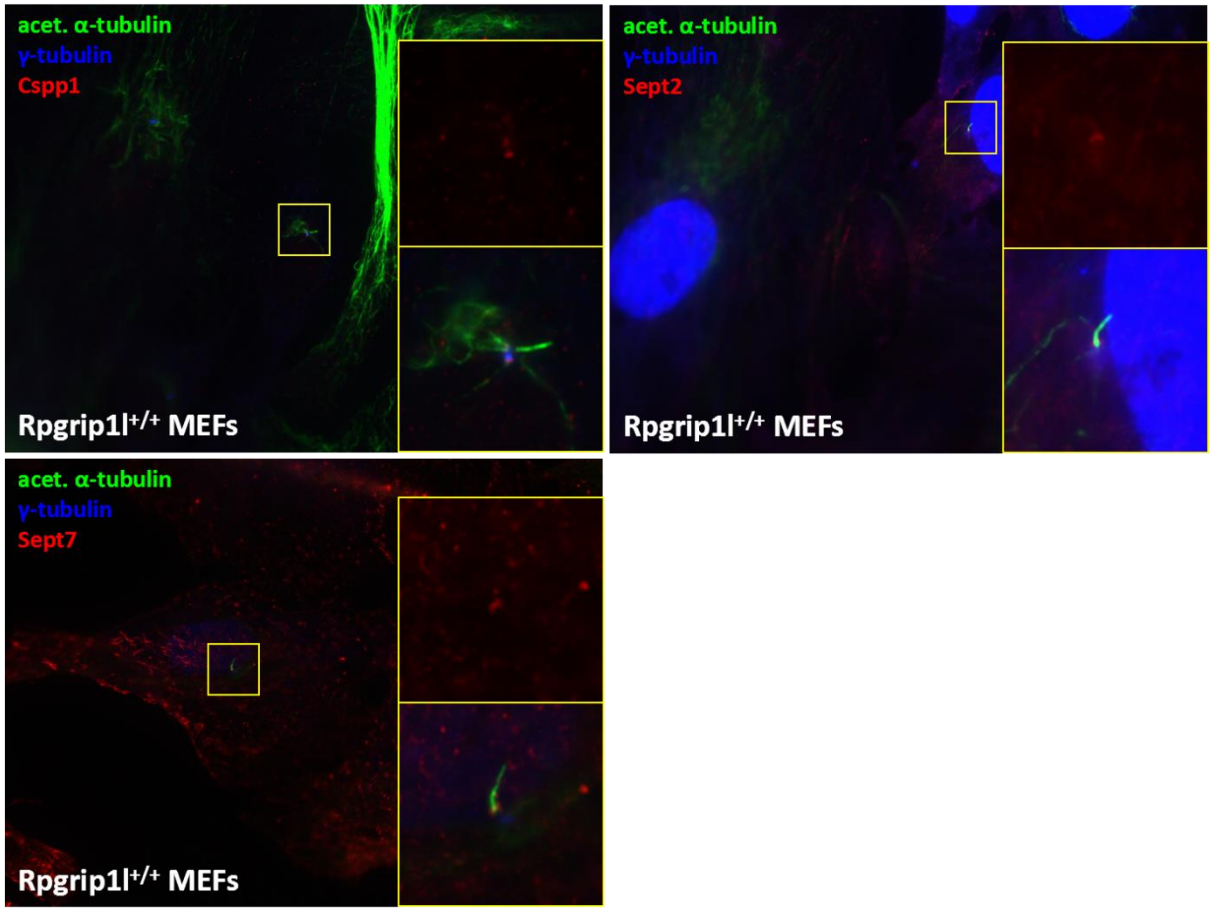
Example of raw images for Figure 1: Measurement of Arl13b and Sstr3 in WT and *Nphp4*$^{-/-}$ MEFs.



Example of raw images for Figure 2: Measurement of Arl13b and Sstr3 in NIH3T3 cells.



Example of raw images for Figure S1: Measurement of Cspp1, Sept2 and Sept7 in WT MEFs.



2. I found hard to judge the accuracy of quantifications. It is not clearly stated how many cells/cilia were quantified in total per condition. In all graphs, if each dot represents one cell/cilia, then the number of cells/cilia analyzed was very low. Please provide the relevant information in each figure legend.

We provided the relevant information in each figure legend. In addition, we adjusted the presentation of our quantification results for Arl13b, Sstr3, Cspp1, Sept2 and Sept7 in MEFs (Figure 1 and Figure S1) to present all measured values of each embryo within a genotype instead of pooled values per genotype. Now, each dot represents one cilium in one cell.

3. Scaffolding function of Rpgrip1l:
The authors show that FLAG-mCep290 binds to the TZ in Rpgrip1l KO cells, whereas a construct carrying Myc-NPHP1 does not. Based on these data, they conclude that Rpgrip1l functions as a scaffold for NPHP1 but not for Cep290. I see a couple of problems with this conclusion. First, the FLAG-mCep290 construct might have a dominant effect (due to the tag and/or mouse variant). Hence, it could constantly bind to the TZ independently of Rpgrip1l, yet endogenous Cep290 might still requires Rpgrip1l as a scaffolding protein. Although, this might be difficult to prove, the authors could at least perform one rescue experiment using different tagged version of Cep290 or non-tagged Cep290 (ideally human Cep290) in Rpgrip1l KO cells.

We performed new transfection experiments in which we transfected a GFP-tagged version of human CEP290 into HEK293 cells as well as the Flag-tagged version of mCep290 into NIH3T3 cells. In both cases, Cep290 accumulates at the ciliary TZ of WT and *Rpgrip1l*$^{-/-}$ cells (Figure S4A, H).

To conclude that Rpgrip1l functions as a scaffold for Nphp1, the authors should show that the Myc-Nphp1 construct is functional and able to bind to the TZ in wild type cells. In figure S3, the authors only show that Myc-Nphp1 does not localize at the TZ in Rpgrip1l null cells. The fact that Myc-Nphp1 is expressed (showed by Western blot), does not mean that it localizes properly.

We thank Referee #1 for mentioning our lack of control in this experiment. We repeated the experiment for WT, *RPGRIP1L*$^{-/-}$ HEK293 and *Rpgrip1l*$^{-/-}$ NIH3T3 cells (Figure S3). In both cell types, transfected Myc-mNphp1 is detectable at the ciliary TZ of WT cells. In contrast to that, no signal is detectable at the TZ in *RPGRIP1L*$^{-/-}$ HEK293 or *Rpgrip1l*$^{-/-}$ NIH3T3 cells, supporting our prior conclusion that Rpgrip1l functions as a scaffold for Nphp1.

4. Based on the rescue of Arl13b localization but not cilia length by FLAG-mCep290, the authors conclude that Rpgrip1l regulates different aspects at the TZ. This assumption is plausible, however, the authors should show that FLAG-mCep290 is fully functional in human cells. For ex., it is known that depletion of Cep290 leads to an increase in ciliary length. Can FLAG-

mCep290 rescues this phenotype? If not, this would imply the FLAG-mCep290 is only partially functional in human cells.

We understand the concerns of Referee #1 regarding the functionality of the pFlag-mCep290 construct in human cells. The answer could be given by the rescue of *CEP290*$^{-/-}$ HEK293 cells by transfecting pFlag-mCep290. However, Cep290-deficient HEK293 cells are not available in our lab. To overcome this limitation, we transfected *Cep290*$^{-/-}$ NIH3T3 cells with the Flag-tagged version of murine Cep290. Consistent with published data [1, 2], our *Cep290*$^{-/-}$ NIH3T3 cells show significantly shorter cilia. The ciliary length was rescued by the transfection of pFlag-mCep290 (Figure S4D), indicating that the pFlag-mCep290 construct is fully functional in NIH3T3 cells. Moreover, we transfected RPGRIP1L-deficient HEK293 cells with a human version of CEP290 (eGFP-hCEP290) (Figure 3D, E and Figure S4H, I), and Rpgrip1l-deficient NIH3T3 cells with the pFlag-mCep290 construct (Figure 3A-C and Figure S4A, B). In both experiments we observed the same results that we had already obtained in our initial analysis: the ciliary amount of Arl13b was restored in *RPGRIP1L*$^{-/-}$ HEK293 cells transfected with eGFP-hCEP290 and in *Rpgrip1l*$^{-/-}$ NIH3T3 cells transfected with pFlag-mCep290 (Figure 3). In both cell types the transfection did not restore ciliary length alterations (Figure S4C, J).

5. The data related to treatment of Rpgrip1 KO cells using eupatilin is interesting. However, I found the conclusion mis-leading. In page 7, the authors state that "The treatment of Rpgrip1l -/- NIH3T3 cells with eupatilin restored the amount of Arl13b and Sstr3 (Figure 4A and B) indicating that Rpgrip1l controls ciliary gating via ensuring the proper amount of Cep290 at the vertebrate TZ." To be able to conclude this, the authors should show that Eupatilin restores Cep290 levels in Rpgrip1l. Can Eupatilin rescues Arl13b localization in Cep290 KO cells? If it does, the results related to Eupatilin would only show that Eupatilin works downstream of Rpgrip1l and Cep290.

We agree with Reviewer #1 that our formulation was ambiguous. Indeed it was previously shown that eupatilin treatment does not restore the level of Cep290 but fulfills its function by indirectly recruiting Nphp5 to the TZ in Cep290 mutant cells [3].
In our study, we show that eupatilin treatment restores Arl13b and Sstr3 ciliary amounts in Rpgrip1l-negative NIH3T3 cells in which the ciliary Cep290 amount is significantly reduced. Therefore, eupatilin restores the ciliary gating in Rpgrip1l-negative cells downstream of Cep290. We also show that transfection of tagged Cep290 constructs into Rpgrip1l-deficient cells restores ciliary gating, which underpins our assertion that Rpgrip1l exerts is gatekeeper function via Cep290. Finally, we show now that the ciliary Nphp5 amount is reduced in Rpgrip1l-negative and Cep290-negative NIH3T3 cells and is restored by eupatilin (Figure 4F-H).
These data lead us to propose that Rpgrip1l exerts its gatekeeper function via controlling Cep290 TZ amounts. Cep290, in turn, controls Nphp5 amounts and thus ciliary gating. In the revised version of our manuscript, we describe this model in more detail in order to avoid any misleading conclusion (Results section pp. 8-9, paragraph “Eupatilin treatment rescues ciliary gating in

Rpgrip1l-negative mouse embryonic fibroblasts" and Discussion section pp. 12-13, paragraph "Rpgrip1l controls ciliary gating via Cep290").

6. In page 10, the authors conclude that "Cspp1 is at the top of the ciliary gating hierarchy involving Rpgrip1l, Cep290 and Nphp5 (Figure 4D)". Could the authors provide experimental evidences for this model? For instance, testing if FLAG-mCep290 and Eupatilin rescue defects of Cspp1 depleted cells. These would be easy to perform experiments that would strengthen the MS.

We agree with Referee #1 that this would be an interesting experiment. Unfortunately, no Cspp1 depleted cells are available in our lab. We revised the text dealing with Cspp1 and the ciliary gating hierarchy (Discussion section, p.12 second paragraph). Moreover, we replaced our simplified model of the proposed gating hierarchy (former Figure 4D) by a general presentation of the ciliary gating status in the different conditions of our study (Figure 5).

Minor points:
1. Please provide data showing the specificity of the antibodies used. This is particularly important for results showing no change in protein localization upon gene depletion.

All of the used antibodies were extensively analyzed before [1, 4-6]. To confirm the Cep290-antibody specificity, we performed a Cep290 staining in WT compared to *Cep290*$^{-/-}$ NIH3T3 cells (Figure S4E, G) [1].

2. Cite papers that previously shown reduction of Arl13b and Ac3 in the absence of Cep290 (e.g. Molinari et al, 2019, Shimada et al., 2017).

We apologize for not citing the mentioned papers in the first version of our manuscript. We included the paper above as well as additional papers dealing with the function of Cep290 in ciliary gating [3, 7-11] (p.12).

3. It is not clear how images were taken (z-stacks? How many? spacing?) and how images were treated for quantifications (quantifications done using sum projections, max. projections?). Please provide detailed information in materials and methods.

We provided more detailed information about Image Processing (p. 23), Image Presentation (p. 23) and Quantification (p. 24) in the Materials and Method section of our revised manuscript.

4. Figure 3D and E, ciliary length. In the images showed in figure 3D, the cilium of Rpgrip1l -/- cells seems to be approx. 3 times longer than the WT cilium. However, the quantifications in 3E show an average ciliary length increase of only 1.5x in Rpgrip1l -/- cells. Please clarify and show a representative image of the average to avoid mis-conception.

We realised the problem of our representative image selection in Figure 3 and thank Referee #1 for pointing that out. We replaced the respective images.

5. It would be more informative if the authors could plot the cilia length measured (in µm) instead of the normalized cilia length in figure 3E.

We agree with Referee #1 and converted all graphs showing ciliary length measurements in order to present ciliary length in µm instead of normalized data.

Reviewer #2 (Remarks to the Author):

In this study, the authors examined the role or RPGRIP1L in ensuring CEP290 proper localization to the TZ to maintain ciliary gating and discuss the therapeutic potential of the flavonoid eupatilin to rescue ciliopathies due to RPGRIP1L mutations. The authors show that Sstr3 is reduced in Rpgrip1l ko cilia. They then go on to show that the ciliary levels of Arl13b and Sstr3 are not affected in NPHP1 and Inversin ko cells, but are reduced in Cep290 inactivated cells.

1. The authors concluded that CEP290 mislocalisation in Rpgrip1l ko cells may underlie the ciliary gating defect. However, CEP290 mislocalisation was not a direct effect of Rpgrip1l loss, as overexpression of FLAG-mCEP290 in Rpgrip1l ko cells rescued the gating defect for Arl13b. This be has the question of how CEP290 is mislocalised in the absence of Rpgrip1l and how such mechanism does not affect the correct localisation of FLAG-mCEP290. Also, what is the reason of using murine and not human CEP290 in these studies, particularly since HEK293 cells are of human origin?

We appreciate this comment of Referee #2 and agree that the question of how the amount of Cep290 at the TZ in absence of Rpgrip1l is regulated needs to be discussed in more detail. We have revised the text and discuss possible explanations in the revised version of our manuscript (pp. 11-12).
In regard to the second question of Referee #2, we performed new transfection experiments. We transfected NIH3T3 cells with the previously used pFlag-mCep290 construct as well as HEK293 cells with an eGFP-tagged version of human CEP290 (Figure 3 and Figure S4). As explained in our response to Reviewer #1's comment 3, we obtained similar results in both conditions.

2. The studies started with MEFs but moved on to NIH3T3 cells. Most of the data and cell lines have been published earlier by this group. Does the effect of CEP290 on ciliary Arl13b and Sstr3 also happen in MEFs?

The role of Cep290 in ciliary gating is conserved across different species and it has been shown for different murine and human cell types that a loss or a reduction of Cep290 leads to disrupted ciliary gating [3, 7, 10]. We started our experiments by comparing ciliary gating in WT and Rpgrip1l-negative MEFs. Afterwards, we aimed to compare ciliary gating function in different ciliary mutants. However, we did not have Cep290-deficient MEFs in the lab. Therefore, we used Nphp4-negative MEFs and several mutant NIH3T3 cells generated via CRISPR/CAS. With these tools we were able to compare WT, Nphp4-negative and Rpgrip1l-negative MEFs as well as WT, Nphp1-negative, Invs-negative, Cep290-negative and Rpgrip1l-negative NIH3T3 cells. Consistent with the fact that NIH3T3 cells are immortalised MEFs, we could not observe any differences between Rpgrip1l-negative MEFs and Rpgrip1l-negative NIH3T3 cells.
Since we have focused on comparing the ciliary gating function of Rpgrip1l and Cep290 in the following, we have performed all further analyses with NIH3T3 cells and not with MEFs.

3. The failure of FLAG-mCEP290 transfection in MEFs has not been explained. Earlier studies have shown efficient transfection of CEP290 into these cells. The switch to HEK293 adds another variable to the experiments. The ectopic staining of recombinant NPHP1 should be provided in the same cell where no effect on ARL13B recruitment was observed.

We fully understand the concerns of Referee #2 regarding the failed transfection experiments. We repeated the experiments and were able to transfect NIH3T3 cells with the Flag-tagged version of mCep290. In addition, we transfected HEK293 cells with a GFP-tagged version of human CEP290 (Figure 3 and Figure S4).
Furthermore, we thank Referee #2 for mentioning our lack of control in the Nphp1 transfection experiment. In the revised version of our manuscript, we have included transfection experiments of pMyc-mNphp1 in WT and *Rpgrip1l*-negative NIH3T3 cells as well as in WT and *RPGRIP1L*$^{-/-}$ HEK293 cells (Figure S3).

4. The mechanism underlying elongated cilia and lack of effect of CEP290 reconstitution on cilia length has not been dealt with at all in this study. The author's group has also previously shown cilia length extension in Rpgrip1l mutant cells and tissues; however, the mechanism is not provided.

We agree with Referee #2 that we have not provided a mechanism underlying the ciliary length alteration in Rpgrip1l-negative cells. Since the regulation of ciliary length is an intensively discussed topic, which is unfortunately not yet well understood in its complexity, it is difficult to find a viable explanation for our observations. Nevertheless, we have discussed our observations on ciliary length variations and possible explanations in more detail in the revised version of our manuscript (Discussion section, pp. 13-15, section “Ciliary length control and ciliary gating is mediated by different mechanisms”).

5. The experiment and conclusions of eupatilin treatment seem preliminary at best. The authors discuss a possible mechanism involving NPHP5; however, given the relatively mild phenotype of NPHP5 mutations, like that of NPHP1 and NPHP4, the involvement of NPHP5-mediated mechanisms in Rpgrip1l ciliary gating seems like a stretch.

We understand that our discussion involving Nphp5 in ciliary gating may seem a little overstretched at first. We revised the corresponding text passage carefully and included an extended discussion about the involvement of Nphp5 in ciliary gating (Discussion section, p. 13, chapter “Rpgrip1l controls ciliary gating via Cep290”). In addition, we performed a Nphp5 staining in eupatilin treated WT, Rpgrip1l-negative and Cep290-negative NIH3T3 cells to clarify our argumentation. We now show that the TZ amount of Nphp5 is strongly reduced in Rpgrip1l-deficient cells and that this is restored by eupatilin treatment.

6. This study is computing in a confusing manner data from MEFs, HEK293 and NIH3T3. If CEP290 overexpression could be a limitation in hard to transfect cells, positive effects of eupatilin treatment should be done in RPGRIIP1L mutant MEFs, in addition to in vivo benefits in the corresponding mice model.

Fortunately, we were now able to transfect NIH3T3 cells with the Flag-tagged version of mCep290 and could therefore perform transfection experiments and drug treatments in the same cell line (Figure 3, Figure 4, Figure S4). With this, we hope to dispel the overall concerns of Referee #2 regarding the use of different cell lines.

We appreciate the idea of Referee #2 to set up a corresponding *in vivo* model. Unfortunately, these experiments are highly time-consuming and data on them cannot be included in the present manuscript. However, we discuss the possibility of an eupatilin treatment in Rpgrip1l-negative mouse embryos in the revised version of our manuscript (Discussion section, p. 13).

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RE: Manuscript #E20-03-0190R
TITLE: "Rpgrip1l controls ciliary gating by ensuring the proper amount of Cep290 at the vertebrate transition zone"

Dear Dr. Gerhardt:

The reviewer has raised the question of sample sizes for some experiments. Would you be able to respond to me to address this so I that can reach a final decision. There may be clear reasons for smaller samples sizes which can be explained by small additions to the text or figure legends. If you do decide to update any figures, then please indicate what changes you have made.

Sincerely,
Francis Barr
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Gerhardt,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
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Reviewer #1 (Remarks to the Author):

The authors addressed most of my concerns. Imaging processing and presentation are now clearly stated in Materials&Methods. My suggestion is to combine the parts related to quantifications and imaging presentation. The title "imaging processing" is misleading as only imaging acquisition is described. Please adjust the title accordingly
The authors also now state the number of cilia analyzed in each experiment. The numbers are very low (10 or 20 cilia per clone for some experiments) and it is not clear how many cilia in total were indeed quantified in all experiments. The authors should state the exact number of cilia analyzed or give a range. There is no mention to the number of biological repetitions that were done per experiment. The low number analyzed is hard to understand because the experiments are based on immunofluorescence staining, which should yield hundred of cells for analysis. Which criteria the authors used to choose 10 cells only? At present, I consider the "n" too low and I raise concerns about the quality of the data presented.

RE: Manuscript #E20-03-0190RR
TITLE: "Rpgrip1l controls ciliary gating by ensuring the proper amount of Cep290 at the vertebrate transition zone"

Dear Dr. Gerhardt:

Thank you for carefully addressing the final questions raised by the reviewer. There are no remaining issues, so I am pleased to be able to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Francis Barr
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Gerhardt:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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