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Rab35 controls cilium length, function and membrane composition

Stefanie Kuhns, Cecília Seixas, Sara Pestana, Bárbara Tavares, Renata Nogueira, Raquel Jacinto, José S. Ramalho, Jeremy C. Simpson, Jens S. Andersen, Arnaud Echard, Susana S. Lopes, Duarte C. Barral, Oliver E. Blacque

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(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.)

1st Editorial Decision 31 January 2019

Thank you for submitting your manuscript for consideration by EMBO Reports. It has now been seen by three referees whose comments are shown below.

As you can see, all referees express interest in the proposed function of Rab35 in regulation of ciliary length and function. However, they also raise concerns that need to be addressed in full before we can consider publication of the manuscript here. In particular, the referees find that currently the study does not provide sufficient mechanistic insight into the role of Rab35 in regulation of ciliary length and function.

Given these constructive comments, I would like to invite you to revise your manuscript with the understanding that the referee must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO Reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS

Referee #1:

This manuscript describes a novel ciliary GTPase, the Rab Rab35. Rab35 is shown to localize to cilia and conditions that elevate Rab35-GTP lead to longer cilia (GAP depletion, overexpression of QL mutant) while conditions that reduce the levels of Rab35-GTP lead to shorter cilia (GEF depletion, overexpression of SN mutant). Reduction in Rab35GTP leads to increases in ciliary ARL13B levels and Rab35 depletion leads to increased ciliary levels of INPP5E and SMO.

The experiments are well-executed and extensively quantitated. The manuscript is sound and solid

and constitutes a valuable addition to the ciliary trafficking literature. As detailed below, a small number of experiments would add considerable impact to the paper and should be considered before publication.

Major comment:

The report is a valuable addition to the literature in its current form but falls short of addressing the mechanistic details behind Rab35-GTP in cilia. While the authors speculate on various roles of Rab35 in regulating endocytosis or IFT function, very little consideration is given to the possibility that Rab35 regulates ectocytosis. This lacuna is all the more surprising when one consider the abundant evidence that endosomal levels of PI45P2 and F-actin are elevated upon depletion of Rab35 (see Klinkert & Echard, 2016) and that elevation of ciliary PI45P2 leads to actin-dependent ciliary decapitation (aka ciliary ectocytosis, Phua Cell 2017). The authors correctly mention that the ciliary 5-phosphatase OCRL1 is a Rab35 effector and it seems logical to predict that ciliary PI45P2 is elevated in the absence of ciliary Rab35GTP.
At the very least, the hypothesis that Rab35 control ciliary length and the abundance of specific ciliary membrane proteins by regulating ectocytosis should be covered to the same extent and with the same level of details as the other hypothesis that are proposed in the discussion. Given the relative ease of detection of ciliary PI45P2 (using either antibodies or PH probes, see Garcia-Gonzalo and Chávez, DevCell15), it would seem reasonable to request to probe for ciliary PI45P2 in cells with altered levels of ciliary Rab35GTP. Testing whether inhibition of ciliary ectocytosis (pharmacological interference with Myo6, drebrin, actin or cilia-targeted Thymosin) leads to restoration of ciliary length would similarly add considerable interest to the paper.

Minor comments:

Fig. 6A: Did the top panels become inverted?

Fig. 8A seems to suggest a role of Rab35 in regulating entry but (as discussed in line 417-475) it is equally likely that Rab35 regulates ciliary exit. In particular, the authors allude to the possibility that Rab35 might regulate ectocytosis but never introduce the concept that ectocytosis is utilized as a means of ciliary exit for ciliary membrane proteins and as a means of cilia shortening.

Line 473 'In support of this notion, Arl13b undergoes IFT, interacts with IFT-B, and requires IFT for its ciliary targeting in C. elegans' Why is Williams et al, 2014 cited here?
Also, it seems odd that Nozaki et al. 2017 is cited without mentioning that this paper found that a mutant of ARL13B defective for IFT-B binding still localized to cilia.

Citation of Ishikawa and Marshall 2011 seems outdated when recent reviews providing broad coverage of ciliary structure and function (Leroux Reiter 2018) or specific coverage of the IFT complexes (Nachury 2018) are available.

The sentence spanning lines 76-77 needs to also include ectocytosis as recent evidence has pointed to this the regulation of ciliary membrane homeostasis by this process,

Line 91-92 needs to add Rab34 to the list of Rabs with functions in ciliary trafficking (Pusapati DevCell18, Xu JCS18).

Line 115-116 should cite the recent CRISPR screens by Pusapati and Breslow as both screens identified Rab35 as a regulator of Hh signaling in 3T3 cells. This comment also applies to line 154-158

Line 143-144. The statement 'In the distal cilium, the GFP-RAB35 signal coincides with that of ARL13B' seems odd in light of Figure 1D where the green signal of Rab35 and the red signal of Arl13B appear largely non-overlapping.

Referee #2:

This is an interesting manuscript that provides data in support of the hypothesis that Rab35, in

addition to its other cellular functions, plays a role in the control of cilia length. The authors are experts on the roles of Rab GTPases in cilia and have recently published in excellent review on the topic (Blacque et al., 2017). Rab35 has been previously associated with primary cilia, although the published data show both positive and negative roles for the protein in cilia formation. Here the authors carry out a systematic study in several cell types and conclude that Rab35 has a positive role in cilia formation and acts through regulation of another small GTPase, ARL13B.

The authors provide high quality data showing that Rab35-GFP localizes to the length of the cilium. Knockdown experiments in RPE1 and IMCD3 cells show a modest but significant shortening of cilia in these cell lines. The statistical analysis of the effect is shown only as error bars in the figure; the actual values here (and throughout the manuscript) should be given in the text. The knockdown appears to be efficient, but knockout cells would be more convincing. If the reason they did not use Crispr/Cas9 to make knockout cell lines is because of the roles of Rab35 in other cellular processes, this should be stated explicitly.

The authors build a consistent case that the reason for the shorter cilia (and ultimately affects on Smo localization to cilia) is because Rab35 is a negative regulator of the ciliary localization of ARL13B.Their arguments are: siRNA to deplete Rab35 causes shorter cilia in RPE and IMCD3 cells; RAB35-S22N (Dominant negative) causes shorter cilia in RPE cells; and RAB35183 Q67L (activated form) leads to longer cilia. From this they conclude that Rab35 is a positive regulator of cilia length.

From previous work, ARL13B is also a positive regulator of cilia length: Arl13b null cilia are short and ARL13B overexpression leads to longer cilia. The authors argue that Rab35 acts through regulation of ARl13b because levels of ciliary ARL13B are elevated 1.5- to 2-fold upon RAB35 depletion (Line 272) and overexpressed Rab35 leads to decreased ciliary Arl13B (Line 289ff). Thus they conclude that Rab35 negatively regulates ARL13B in the ciliary membrane.

However, the argument is complex: Rab35 depletion leads to elevated levels of ciliary ARL13B (Fig 5 and Line 272) and shorter cilia, whereas elevation of ARL13B, in a number of other people's hands, causes longer cilia. Indeed, in the Discussion the authors state that there is no correlation between cilium length and ARL13B levels.

This apparent contradiction highlights the absence of data about a possible mechanism connecting Rab35 and ARL13B. One interpretation of the findings might predict that expressing activated ARL13B would rescue the Rab35 knockdown phenotype. One might also expect that the proteins would be present in the same protein complex. Given the confusing nature of their findings, the absence of genetic or proteomic support for their model makes it difficult to accept their conclusions.

The authors also attempt to document the same effects in zebrafish, but these experiments have even more problems. Using injection of translation-blocking morpholinos, they knock down expression of Rab35, but the level of knockdown is not quantitated. They state that the fish show a set of phenotypes that resemble those of cilia mutants, but only show one image of a whole embryo or animal in the supplemental material. This animal does not look like typical zebrafish cilia mutant; the most prominent phenotype of this animal is whole body edema, a non-specific effect that could easily be due to other targets of Rab35. To document a situs defect, they present data on counting the position of the liver and heart, but no raw data are shown. They need to show images of the hearts to determine whether the phenotype could be caused by an early edema that affects the heart as well as other tissues. Most importantly, they need to examine the expression of left/right marker genes to show the specificity of the phenotype.

This skepticism about the zebrafish phenotype is reinforced by the absence of a cilia phenotype in Rab35 null mice. While there is no data about cilia phenotypes in Rab35 null animals, the Rab35 null mouse phenotype is very mild, in contrast to the strong phenotypes shown by cilia mutants such as Arl13b. Rab35 null mice are viable, and have defects in aging, reproduction and in the eye. It would be worthwhile for the authors to obtain null mice or cell lines to examine whether Rab35 has subtle effect on cilia in the mouse. However the burden of proof is on the authors.

Other comments:

The error bars on cilia length measurements seem large; what do the raw numbers look like? The authors should state the numbers on the percentage or fold change and statistical significance in the text. (e.g. Lines 164 Line254 261-263 etc.

All localization studies are done with a GFP tagged protein. Have the authors attempted to examine the localization of endogenous Rab35? This would substantially enhance the manuscript.

The authors should use a centriole marker or nuclei staining together with acetyl-α-tubulin to quantify the fraction of ciliated cells in the zebrafish experiments (Figure 4).

Line 202-204. "For DENND1C and TBC1D10C, no expression was detected in hTERT-RPE1 cells, and therefore they were excluded from subsequent experiments." What work is this based on? Please cite. What is the expression pattern of these regulators of Rab35 in IMCD3 cells; is it conserved?

Line 270: "its established role in regulating protein levels at the plasma membrane" Please add reference and include in the introduction.

Figure 4A. The RAB35-mCherry staining is very faint. The images should be improved.

Figure 6A. The anti-Flag immunoblotting panels (top) are reversed between input and IP experiment.

Referee #3:

In this paper, the authors showed that Rab35, a regulator of endosomal trafficking, plays a role in controlling cilium length and the composition of ciliary membrane proteins.
The authors found that Rab35 localizes to the cilium and that cilia were much shorter with altered levels of ciliary membrane proteins in Rab35 depleted cells. The authors confirmed these results in vivo using zebrafish, and further showed that the animal displayed ciliopathy-related phenotypes.

In addition to the novelty of the result, this paper is well written and most of experiments were carefully performed. The data quality is also high. I also appreciate that the authors provided a good amount of information to ensure reproducibility in the materials and methods. I believe most of the results that the authors presented in this paper.

A weak point of this paper is that a molecular mechanism by which Rab35 regulates ciliary length is unclear. So, I would appreciate if authors could address the following points.

Major point

1) Authors showed that GFP-Rab35 localizes to ciliary pocket in addition to its ciliary localization. As authors mentioned in discussion, ciliary pocket localization of Rab35 is quite intriguing, because Rab35 is known to be involved in endocytic trafficking pathway and ciliary pocket is a site of endocytosis. Therefore, it seems to be very important to dig into the localization a little more deeply. Authors claimed that Rab35 localizes to ciliary membrane because it co-localizes with ARL13B in the distal portion of cilium (line 143-145). However, ciliary pocket membrane is distant from ciliary membrane at the proximal cilium, whereas ciliary pocket membrane is in close proximity to ciliary membrane at the distal cilium (see for example http://jcs.biologists.org/content/123/10/1785). Furthermore, authors showed that GFP-Rab35 was concentrated in the proximal region of cilium (Fig. 1C, 1D, and S1). Therefore, Rab35 might be exclusively localized to ciliary pocket membrane.

The best way to perfectly distinguish ciliary pocket localization from ciliary membrane localization would be immuno-gold electron microscopy, but I understand that this approach is too much for this paper. Alternatively, it would be great if authors could test if Rab35 colocalizes with EHD1 using either EHD1 antibody or GFP-EHD1. It would also be informative if authors could show what percentage of cells have Rab35 along the entire cilium (like Fig.1C) and what percentage of cells have Rab35 in a portion of the cilium (like Fig 1D, right). Comparing those data with the previous

report (Fig. 3d of https://ciliajournal.biomedcentral.com/articles/10.1186/s13630-016-0044-2) would be interesting.

Also, authors should characterize the localization of DENND1B and TBC1D10A more deeply (Fig. 3H and 3I). Do these proteins localize to the ciliary pocket? What percentage of cells have ciliary/centrosomal DENND1B/TBC1D10A? Where at centrosome does TBC1D10A localize? Distal appendage? Sub-distal appendage? Or daughter centriole?

2) To understand how Rab35 regulates ciliary membrane composition, finding its downstream effector would be essential. Given that ARL13B interacts with Rab35, ARL13B might be an effector protein (or an interactor of an effector protein). It seems like the authors sought to answer the question (Fig. 6D-F), however, these experiments were not properly designed.
In these experiments, GDP/GTPγS was added to cell lysates to promote GDP/GTPγS binding to Rab35/ARL13B. I suppose that most of Rab35 proteins do not bind to newly added GDP/GTPγS in this experimental condition. A typical Rab GTPase has very high affinity for guanine nucleotide (Kd in low nM to pM level), thus needs GEF to lower its nucleotide affinity to allow for its activation (GTP-binding). In vitro experiment, nucleotide exchange is often performed in the presence of EDTA, which chelates magnesium ion to lower the affinity for GDP/GTP. In the experimental condition used by the authors, Rab35 should not bind efficiently, if any, to newly added guanine nucleotides. In contrast, ARL13B has relatively lower affinity for guanine nucleotide (equilibrium dissociation constant for GppNHp is 0.4 μM according to the previous report http://www.jbc.org/content/292/26/11091). ARL13B should be able to undergo nucleotide exchange without the need for GEF. So, the enhancement of the binding between ARL13B and Rab35 (Fig. 6D) seems to come from GTP binding to ARL13B rather than Rab35.
In addition, ARL13B (G28V) used in Fig. 6E should not work as a GTP-locked mutant. I assume that authors in the previous paper (Dukdulao et al., 2009) tried to make a GTP-locked mutant that corresponds to Kras G12V. Glycine 12 of Kras is located at X2 position of GX1X2X3X4GKS/T motif (G1 loop). G1 loop is GLDNAGKT in human ARL13B, and substitution of invariant glycine to valine should lower affinity of ARL13B for GDP/GTP, rather than locking it at the GTP-bound state.

Authors should simply perform a co-IP experiment with the use of GDP-locked (S22N) and GTP-locked (Q67L) mutant of Rab35 to test the hypothesis.

Minor point

1) line 179-181. "We found that RAB35-S22N overexpression exerts a dominant-negative effect on ciliogenesis, resulting in a severe reduction in ciliation (down to 25% of cells) (Fig 3C)"

The ciliation phenotype of cells expressing Rab35-S22N is apparently different from that of Rab35 depleted cells (Fig. 2B). The authors should comment how this difference occurs.

2) line 219-220. "localisation was observed for DENND1A or TBC1D10B (Fig 3G and H, and Appendix Fig S4C and D)."

"Fig. 3G and 3H" should be "Fig. 3H and I".

3) line 246-247. "compared with 1 and 10%, of non-injected and mismatch MO control embryos, respectively."

Does this sentence correspond to Fig. S5B? The data for mismatch MO is missing.

4) line 254. "mismatch MO-injected or non-injected embryo controls (Fig 4D and E)."

Data for non-injected control is missing.

5) line 261-263. "We observed that KV cilia length is significantly reduced in embryos overexpressing Rab35-S22N, when compared with the overexpression of Rab35-WT or mCherry alone (Fig 4F-G)"

Authors should mention whether cilia are longer in embryos expressing Rab35-Q67L. If there is no difference between control and Q67L, it should be indicated in the figure (Fig. 6G).

6) lind 269-270. "its established role in regulating protein levels at the plasma membrane"

Refences for this statement is missing.

7) line 512. "HEK239T" should be "HEK293T".

8) line 515. "Cilia formation in hTERT-RPE1, IMCD3 or HEK293T"

To help readers to reproduce the data, it would be great if authors could describe how many cells were plated in which plate.

9) line 519 "HEK293T cells were transiently transfected with plasmid DNA using the calcium phosphate precipitation method"

How many cells were plated? Please describe calcium phosphate precipitation method in a little more detail.

10) line 522, 524, 526, 533. "TransIT-LT1" "Lipofectamine 3000" "Lipofectamine 2000"

What amount of the reagent and plasmid DNA (or siRNA) was used for transfection?

11) line 662-669. "HEK293T cells and hTERT-RPE1 cells were lysed at 4{degree sign}C for 20 min - proteins were eluted in Laemmli sample buffer."

For co-IP experiments, how many cells were plated in which plate? What concentration of cell lysates were used for co-IP? What is the total volume of co-IP reaction? What amount of beads was used for co-IP?

12) line 669-673. "For immunoprecipitation with GTPγS -and immunoprecipitations was performed for 2 h"

Final concentration of GDP/GTPγS is missing. What concentration of cell lysates were used? Immunoprecipitation performed at 4°C? No magnesium added in the buffer?

13) Fig. 1C. In line profile plots, each color contains three lines (one is a solid line and the others are dotted lines). Please explain what those three lines are in the figure legend.

14) Fig. 2D. Authors generated a clonal cell line expressing GFP-Rab35 for the rescue experiment. Clonal cell line might have different characteristics from the parent cell line. Indeed, cilium length of GFP-Rab35 expressing cells looks shorter than control cells. Similarly, knockdown efficiency might be different between control cells and GFP-Rab35 expressing cells. Authors should show Western blot to confirm that knockdown efficiency is comparable between the two lines. Also, authors should show the expression level of GFP-Rab35 compared with endogenous Rab35.

15) Fig. 3A. Did author use polyglutamylated tubulin? Or acetylated tubulin? The leftmost panel was labeled with polyglutamylated tubulin, but the smaller panels were labeled with acetylated tubulin.

16) Fig. 6A.
A) The data for input and IP is flipped.
B) Authors should mention what percentage of input was used.
C) Ideally, input and IP should be loaded side by side on the same gel, blotted on the same membrane, and detected with the same exposure time. This way allows readers to estimate how strong the interaction is and help them to reproduce the data. Authors should at least mention whether the samples were loaded on the same gel, blotted on the same membrane, and detected with the same exposure time.

17) Fig. 6C. "ARL13B V385A-GFP" should be "ARL13B V358A-GFP".

18) Fig. 6D and 6F. Authors should comment why the interaction between Rab35 and ARL13B decreased in the presence of GDP in Fig.6D but increased in Fig. 6F.

19) Fig. 7C. Authors should perform Western blot to confirm that knockdown of Rab35 and ARL13B in Rab35/ARL13B double depleted cells was as efficient as either Rab35 or ARL13B single depleted cells. Also, authors should mention that how the double knockdown was performed.

20) Table S1. "Human ARL13B (V385A)" should be "Human ARL13B (V358A)"

21) Table S2. A catalog number for polyclonal rabbit anti-RAB35 from Proteintech should be "11329-2-AP".

1st Revision - authors' response 24 June 2019

Referee #1:

This manuscript describes a novel ciliary GTPase, the Rab Rab35. Rab35 is shown to localize to cilia and conditions that elevate Rab35-GTP lead to longer cilia (GAP depletion, overexpression of QL mutant) while conditions that reduce the levels of Rab35-GTP lead to shorter cilia (GEF depletion, overexpression of SN mutant). Reduction in Rab35GTP leads to increases in ciliary ARL13B levels and Rab35 depletion leads to increased ciliary levels of INPP5E and SMO.

The experiments are well-executed and extensively quantitated. The manuscript is sound and solid and constitutes a valuable addition to the ciliary trafficking literature. As detailed below, a small number of experiments would add considerable impact to the paper and should be considered before publication.

Major comment:

The report is a valuable addition to the literature in its current form but falls short of addressing the mechanistic details behind Rab35-GTP in cilia. While the authors speculate on various roles of Rab35 in regulating endocytosis or IFT function, very little consideration is given to the possibility that Rab35 regulates ectocytosis. This lacuna is all the more surprising when one consider the abundant evidence that endosomal levels of PI45P2 and F-actin are elevated upon depletion of Rab35 (see Klinkert & Echard, 2016) and that elevation of ciliary PI45P2 leads to actin-dependent ciliary decapitation (aka ciliary ectocytosis, Phua Cell 2017). The authors correctly mention that the ciliary 5-phosphatase OCRL1 is a Rab35 effector and it seems logical to predict that ciliary PI45P2 is elevated in the absence of ciliary Rab35GTP. At the very least, the hypothesis that Rab35 control ciliary length and the abundance of specific ciliary membrane proteins by regulating ectocytosis should be covered to the same extent and with the same level of details as the other hypothesis that are proposed in the discussion. Given the relative ease of detection of ciliary PI45P2 (using either antibodies or PH probes, see Garcia-Gonzalo and Chávez, DevCell15), it would seem reasonable to request to probe for ciliary PI45P2 in cells with altered levels of ciliary Rab35GTP. Testing whether inhibition of ciliary ectocytosis (pharmacological interference with Myo6, drebrin, actin or cilia-targeted Thymosin) leads to restoration of ciliary length would similarly add considerable interest to the paper.

Using a PIP2 sensor, we have now assessed PI(4,5)P_2 ciliary signals in Rab35 knockout NIH3T3 fibroblasts. In WT NIH3T3 cells, we find PI(4,5)P_2 signal in the vast majority (~80%) of cilia. However, in the Rab35 k/o cells, only ~55% of cilia are positive for PI(4,5)P_2 (see new Figure 7). This observation agrees with the increased INPP5E ciliary levels that we observe in Rab35 disrupted cells, and strengthens our conclusions based on the ARL13B/INPP5E observations.

Since PI(4,5)P_2 was recently reported to promote ciliary excision/ectocytosis (Phua et al. 2017), the reduced ciliary PI(4,5)P_2 levels we observe in Rab35-disrupted cells argues against a model that the Arl13b and ciliary length phenotypes are due to abnormal upregulation of ectocytosis events. Nonetheless, we agree with the reviewer that this possibility

should be explored in the discussion, and thus we have added some text on this theme to the 3rd last paragraph.

We agree that it would be nice to investigate the effect of ectocytosis inhibition on cilium length in Rab35-disrupted cells. However, given our latest $PI(4,5)P_2$ findings, we respectfully submit that this analysis is not essential for the current study.

Minor comments:

Fig. 6A: Did the top panels become inverted?

Now corrected.

Fig. 8A seems to suggest a role of Rab35 in regulating entry but (as discussed in line 417-475) it is equally likely that Rab35 regulates ciliary exit. In particular, the authors allude to the possibility that Rab35 might regulate ectocytosis but never introduce the concept that ectocytosis is utilized as a means of ciliary exit for ciliary membrane proteins and as a means of cilia shortening.

The reviewer is fully correct with this comment. As mentioned above for the major comment, we have now added a section in the discussion that addresses an ectocytosis model.

Line 473 'In support of this notion, Arl13b undergoes IFT, interacts with IFT-B, and requires IFT for its ciliary targeting in C. elegans' Why is Williams et al, 2014 cited here?

We cited Williams *et al.* 2014 because that study showed that mammalian ARL13B undergoes IFT in olfactory sensory cilia.

Also, it seems odd that Nozaki et al. 2017 is cited without mentioning that this paper found that a mutant of ARL13B defective for IFT-B binding still localized to cilia.

We cited the paper because it shows that ARL13B biochemically interacts with IFT machinery. Whilst a mutant of ARL13B is defective for IFT-B binding, this does not preclude the possibility that this ARL13B variant could get into cilia via interactions with other components of the IFT machinery (*e.g.* IFT-A).

Citation of Ishikawa and Marshall 2011 seems outdated when recent reviews providing broad coverage of ciliary structure and function (Leroux Reiter 2018) or specific coverage of the IFT complexes (Nachury 2018) are available.

We agree that we should have cited more recent reviews, and have now done so in paragraph 2 of the introduction.

The sentence spanning lines 76-77 needs to also include ectocytosis as recent evidence has pointed to this the regulation of ciliary membrane homeostasis by this process,

We added this concept to paragraph 2 of the introduction.

Line 91-92 needs to add Rab34 to the list of Rabs with functions in ciliary trafficking (Pusapati DevCell18, Xu JCS18).

We have now added Rab34 to the list in the revised introduction, as suggested.

Line 115-116 should cite the recent CRISPR screens by Pusapati and Breslow as both screens identified Rab35 as a regulator of Hh signaling in 3T3 cells. This comment also applies to line 154-158.

We thank the reviewer for pointing out these important observations, which are now referred to in the suggested parts of the introduction and results sections.

Line 143-144. The statement 'In the distal cilium, the GFP-RAB35 signal coincides with that of ARL13B' seems odd in light of Figure 1D where the green signal of Rab35 and the red signal of Arl13B appear largely non-overlapping.

We presume the reviewer is referring to the right hand set of images in Figure 1D where the distal portion of the cilium is only very weakly staining for GFP-RAB35. We included this image as a good example of a cilium where the proximal-most GFP-RAB35 signal is proximal to - and radially broader than - the ARL13B signal. Nonetheless, to avoid confusion we have rephrased the sentence, removing the 'In the distal cilium' portion. We also draw the reviewer's attention to the line scan quantifications of the GFP-RAB35 and ARL13B signals in Figure 1C and Figure 1E.

Referee #2:

This is an interesting manuscript that provides data in support of the hypothesis that Rab35, in addition to its other cellular functions, plays a role in the control of cilia length. The authors are experts on the roles of Rab GTPases in cilia and have recently published in excellent review on the topic (Blacque et al., 2017). Rab35 has been previously associated with primary cilia, although the published data show both positive and negative roles for the protein in cilia formation. Here the authors carry out a systematic study in several cell types and conclude that Rab35 has a positive role in cilia formation and acts through regulation of another small GTPase, ARL13B.

The authors provide high quality data showing that Rab35-GFP localizes to the length of the cilium. Knockdown experiments in RPE1 and IMCD3 cells show a modest but significant shortening of cilia in these cell lines. The statistical analysis of the effect is shown only as error bars in the figure; the actual values here (and throughout the manuscript) should be given in the text.

We have added p-value numbers to the text. Also, please note that we don't show error bars in the cilium length graphs; these 'bars' are whiskers of a box plot that indicate minimum and maximum values (described also in the legend).

The knockdown appears to be efficient, but knockout cells would be more convincing. If the reason they did not use Crispr/Cas9 to make knockout cell lines is because of the roles of Rab35 in other cellular processes, this should be stated explicitly. The authors build a consistent case that the reason for the shorter cilia (and ultimately affects on Smo localization to cilia) is because Rab35 is a negative regulator of the ciliary localization of ARL13B.Their arguments are: siRNA to deplete Rab35 causes shorter cilia in RPE and IMCD3 cells; RAB35-S22N (Dominant negative) causes shorter cilia in RPE cells; and RAB35183 Q67L (activated form) leads to longer cilia. From this they conclude that Rab35 is a positive regulator of cilia length.

We have made an NIH3T3 knockout cell line for Rab35 using CRISPR/Cas9. Like Rab35-depleted hTERT-RPE1 cells, Rab35 k/o cells (2 lines) display truncated cilia, although the incidence of cilium formation is not affected (see revised Figure 2 I-L). Thus, we show that Rab35 is a positive regulator of cilium length by using two different methodologies to disrupt Rab35 (siRNA depletion & knockout).

From previous work, ARL13B is also a positive regulator of cilia length: Arl13b null cilia are short and ARL13B overexpression leads to longer cilia. The authors argue that Rab35 acts through regulation of ARl13b because levels of ciliary ARL13B are elevated 1.5- to 2-fold upon RAB35 depletion (Line 272) and overexpressed Rab35 leads to decreased ciliary Arl13B (Line 289ff). Thus they conclude that Rab35 negatively regulates ARL13B in the ciliary membrane.

The results with the new Rab35 knockout NIH3T3 cell line data support the observations from Rab35-depleted cells, namely an increase in ciliary Arl13b levels in knockout cells compared to WT controls (see new Fig 5D-F). This new data supports our conclusion that Rab35 negatively regulates the levels of Arl13b at the ciliary membrane.

However, the argument is complex: Rab35 depletion leads to elevated levels of ciliary ARL13B (Fig 5 and Line 272) and shorter cilia, whereas elevation of ARL13B, in a number of other people's hands, causes longer cilia. Indeed, in the Discussion the authors state that there is no correlation between cilium length and ARL13B levels. This apparent contradiction highlights the absence of data about a possible mechanism connecting Rab35 and ARL13B. One interpretation of the findings might predict that expressing activated ARL13B would rescue the Rab35 knockdown phenotype. One might also expect that the proteins would be present in the same protein complex. Given the confusing nature of their findings, the absence of genetic or proteomic support for their model makes it difficult to accept their conclusions.

It is certainly the case that there is an apparent contradiction in the literature regarding whether Arl13b is a positive or negative regulator of cilium length. Of course, the various observations could be at least partially reconciled by cell type-specific functions for Arl13b and the potential for different outcomes to cilium length depending on the level of Arl13b enrichment at the ciliary membrane. Indeed, it should be noted that observations of cilium elongation have been made in cells where Arl13b is overexpressed to a degree that is

likely much higher than the increased endogenous Arl13b ciliary levels we observe in Rab35-disrupted cells. Thus, whilst we agree with the reviewer that it is difficult to reconcile the Arl13b/cilium length phenotype in Rab35 depleted and knockout cells with what is shown in the broader literature, the apparent contradiction in the literature does not by itself invalidate our findings that Rab35 is a positive regulator of cilium length and a negative regulator of ciliary Arl13b levels. Furthermore, our manuscript does not try to rationalise all of the observed phenotypes (cilium length, Arl13b, ciliary Smo) within a singular model of Rab35 function.

It is also possible that the effects we see in Rab35-disrupted cells on cilium length and Arl13b are due to Rab35 functioning in more then one cilia-related pathway. Indeed, in support of the latter, we investigated if there is a correlation between ciliary ARL13B levels and cilium length, but found no such correlation (Fig EV5A). Thus, we now state in our paper that "Additionally, Rab35 disruption may impact more than one cilia-related pathway and, thus, the cilium length defect in cells lacking Rab35 may not arise directly from the elevated ciliary Arl13B levels."

Finally, we must point out that Figure 6 does indeed show a biochemical association between Arl13b and Rab35, contrary to the reviewer's assertion that such data is lacking in the manuscript.

The authors also attempt to document the same effects in zebrafish, but these experiments have even more problems. Using injection of translation-blocking morpholinos, they knock down expression of Rab35, but the level of knockdown is not quantitated.

We have now quantified the decrease in protein level depicted in the western blot (see new Figure 4C).

They state that the fish show a set of phenotypes that resemble those of cilia mutants, but only show one image of a whole embryo or animal in the supplemental material. This animal does not look like typical zebrafish cilia mutant; the most prominent phenotype of this animal is whole body edema, a non-specific effect that could easily be due to other targets of Rab35. To document a situs defect, they present data on counting the position of the liver and heart, but no raw data are shown. They need to show images of the hearts to determine whether the phenotype could be caused by an early edema that affects the heart as well as other tissues.

We show new images of whole embryos at 30 and 48 hpf where the heart positioning can be better appreciated (new Figure EV3). Importantly, it can be observed that the definition of the heart positioning occurs earlier than the edema, when this is present.

We have also simplified the data regarding morphological defects of Rab35 morphants such as curved tail and microphthalmia since their link to cilia-associated phenotypes is less specific. We maintain the pericardial edema since they could be indicative of defects on the pronephros cilia. This is now shown in Figure EV3.

Most importantly, they need to examine the expression of left/right marker genes to show the specificity of the phenotype.

Following the reviewer's suggestion, we now show the localization of dand5 in Rab35 MO and controls at 10 hpf. The quantification shows 45% of bilateral dand5 in Rab35 MO, compared with 0% or 18% in non-injected or mismatch MO, respectively. These results are now shown in Figure 4E- F.

This skepticism about the zebrafish phenotype is reinforced by the absence of a cilia phenotype in Rab35 null mice. While there is no data about cilia phenotypes in Rab35 null animals, the Rab35 null mouse phenotype is very mild, in contrast to the strong phenotypes shown by cilia mutants such as Arl13b. Rab35 null mice are viable, and have defects in aging, reproduction and in the eye. It would be worthwhile for the authors to obtain null mice or cell lines to examine whether Rab35 has subtle effect on cilia in the mouse. However the burden of proof is on the authors.

We are unclear as to the source of this information, as to our knowledge, there is no publication regarding a viable Rab35 null mouse. Indeed, there is information on an EUCOMM-generated Rab35 mouse (MGI:1924657) that is pre-weaning lethal (see https://www.mousephenotype.org/data/genes/MGI:1924657). Also, we were recently in touch with Jeremy Reiter and Brad Yoder (UCSF) whose lab has generated a Rab35 knockout mouse. They have given us permission to relay that their mice are pre-weaning lethal. Thus, it appears that Rab35 is indeed an essential gene.

Other comments:

The error bars on cilia length measurements seem large; what do the raw numbers look like? The authors should state the numbers on the percentage or fold change and statistical significance in the text. (e.g. Lines 164 Line 254 261-263 etc.

These are not error bars, but whiskers of a box plot (details of the box horizontal lines and the extent of the whiskers are in the figure legends). As commented on above, we have nonetheless added p-values to the text.

All localization studies are done with a GFP tagged protein. Have the authors attempted to examine the localization of endogenous Rab35? This would substantially enhance the manuscript.

We agree that localisation data for endogenous Rab35 would be very useful. However, available Rab35 antibodies are not suitable for immunohistochemistry (which is the case for many Rab antibodies). We could try knock-in of GFP 5' to the Rab35 locus via CRISPR/Cas9, but this experimentation is technically very challenging and not well established. We hope that the reviewer can appreciate that we have shown ciliary localisation for GFP-Rab35 in multiple cells, transiently and stabling expressing the GFP-Rab35 construct.

The authors should use a centriole marker or nuclei staining together with acetyl-α-tubulin to quantify the fraction of ciliated cells in the zebrafish experiments (Figure 4).

We agree with the reviewer's point and have now quantified the number of cilia per KV in different zebrafish experiments of Figure 4. We found no significant difference, suggesting that there is no defect in ciliogenesis (new Appendix Figure S3E). This is in agreement with the results we show with mammalian cell lines.

Line 202-204. "For DENND1C and TBC1D10C, no expression was detected in hTERT-RPE1 cells, and therefore they were excluded from subsequent experiments." What work is this based on? Please cite. What is the expression pattern of these regulators of Rab35 in IMCD3 cells; is it conserved?

The expression data for DENND1C and TBC1D10C in hTERT-RPE1 cells is our work, and we now make this clear in the results.

Line 270: "its established role in regulating protein levels at the plasma membrane" Please add reference and include in the introduction.

There is substantial evidence that Rab35 regulates fast recycling pathways from endosomal compartments to the PM; however, from the literature, it is unclear as to what extent PM levels of the cycling proteins are affected in Rab35-disrupted cells. For this reason, we removed the "established role in regulating protein levels at the plasma membrane" part of the sentence in the results.

Figure 4A. The RAB35-mCherry staining is very faint. The images should be improved.

We now show new images in Figure 4A for the detection of Rab35-mCherry in KV cilia. The weak staining can be explained by a transient localization of mCherry-Rab35 in cilia compared to a signal such as for acetylated tubulin. This is in agreement with our data on mammalian cell lines overexpressing Rab35 in which only 60% of cilia have detectable Rab35. Adding to this, there is also difficulty in imaging inside the KV in a whole mounted embryo.

Figure 6A. The anti-Flag immunoblotting panels (top) are reversed between input and IP experiment.

This mistake has now been corrected. See revised Fig 6A.

Referee #3:

In this paper, the authors showed that Rab35, a regulator of endosomal trafficking, plays a role in controlling cilium length and the composition of ciliary membrane proteins.

The authors found that Rab35 localizes to the cilium and that cilia were much shorter with altered levels of ciliary membrane proteins in Rab35 depleted cells. The authors confirmed these results in vivo using zebrafish, and further showed that the animal displayed ciliopathy-related phenotypes.

In addition to the novelty of the result, this paper is well written and most of experiments were carefully performed. The data quality is also high. I also appreciate that the authors provided a good amount of information to ensure reproducibility in the materials and methods. I believe most of the results that the authors presented in this paper.

A weak point of this paper is that a molecular mechanism by which Rab35 regulates ciliary length is unclear. So, I would appreciate if authors could address the following points.

Major point

1) Authors showed that GFP-Rab35 localizes to ciliary pocket in addition to its ciliary localization. As authors mentioned in discussion, ciliary pocket localization of Rab35 is quite intriguing, because Rab35 is known to be involved in endocytic trafficking pathway and ciliary pocket is a site of endocytosis. Therefore, it seems to be very important to dig into the localization a little more deeply. Authors claimed that Rab35 localizes to ciliary membrane because it co-localizes with ARL13B in the distal portion of cilium (line 143-145). However, ciliary pocket membrane is distant from ciliary membrane at the proximal cilium, whereas ciliary pocket membrane is in close proximity to ciliary membrane at the distal cilium (see for example http://jcs.biologists.org/content/123/10/1785). Furthermore, authors showed that GFP-Rab35 was concentrated in the proximal region of cilium (Fig. 1C, 1D, and S1). Therefore, Rab35 might be exclusively localized to ciliary pocket membrane. The best way to perfectly distinguish ciliary pocket localization from ciliary membrane localization would be immuno-gold electron microscopy, but I understand that this approach is too much for this paper. Alternatively, it would be great if authors could test if Rab35 colocalizes with EHD1 using either EHD1 antibody or GFP-EHD1.

We also observed ciliary localisation of GFP-Rab35 in IMCD3 cells, a cell line that rarely forms a ciliary pocket. Nonetheless, as suggested, we costained GFP-RAB35 expressing hTERT-RPE1 cells for endogenous EHD1. The data (now presented in Figure 1E) shows that EHD1 signals colocalise with GFP-RAB35 in the proximal region of the cilium; importantly, the radial extent of the RAB35/EHD1 signal is broader than that of the ARL13B signal at the ciliary membrane, which is highly suggestive of RAB35 localization to the ciliary pocket, as well as the ciliary membrane.

It would also be informative if authors could show what percentage of cells have Rab35 along the entire cilium (like Fig.1C) and what percentage of cells have Rab35 in a portion of the cilium (like Fig 1D, right). Comparing those data with the previous report (Fig. 3d of https://ciliajournal.biomedcentral.com/articles/10.1186/s13630-016-0044-2) would be interesting.

We quantified the % of cilia with RAB35 along the entire and proximal cilium, referenced to ARL13B and polyglutamylated tubulin staining (see Figure EV1C). For RAB35-positive cilia, ~70% show RAB35 along the entire cilium length (as marked by ARL13B) and ~ 30% show RAB35 only within a proximal domain.

Also, authors should characterize the localization of DENND1B and TBC1D10A more deeply (Fig. 3H and 3I). Do these proteins localize to the ciliary pocket? What percentage of cells have ciliary/centrosomal DENND1B/TBC1D10A? Where at centrosome does TBC1D10A localize? Distal appendage? Sub-distal appendage? Or daughter centriole?

We now provide quantification of the GFP-DENND1B and GFP-TBC1D10A ciliary localisations in Figure 3J. The data shows that ~45% of cilia are positive for these markers. When localised to the cilium, GFP-DENND1B is found ~85% of the time in the ciliary axoneme and only 15% of the time at the ciliary base. The reverse is true for GFP-TBC1D10A-positive cilia, where the marker is mostly present at the ciliary base (90% of cases), with only a small minority (~10%) showing ciliary base localisation.

2) To understand how Rab35 regulates ciliary membrane composition, finding its downstream effector would be essential. Given that ARL13B interacts with Rab35, ARL13B might be an effector protein (or an interactor of an effector protein). It seems like the authors sought to answer the question (Fig. 6D-F), however, these experiments were not properly designed. In these

experiments, GDP/GTPS was added to cell lysates to promote GDP/GTPS binding to Rab35/ARL13B. I suppose that most of Rab35 proteins do not bind to newly added GDP/GTPS in this experimental condition. A typical Rab GTPase has very high affinity for guanine nucleotide (Kd in low nM to pM level), thus needs GEF to lower its nucleotide affinity to allow for its activation (GTP-binding). In vitro experiment, nucleotide exchange is often performed in the presence of EDTA, which chelates magnesium ion to lower the affinity for GDP/GTP. In the experimental condition used by the authors, Rab35 should not bind efficiently, if any, to newly added guanine nucleotides. In contrast, ARL13B has relatively lower affinity for guanine nucleotide (equilibrium dissociation constant for GppNHp is 0.4 μM according to the previous reporthttp://www.jbc.org/content/292/26/11091). ARL13B should be able to undergo nucleotide exchange without the need for GEF. So, the enhancement of the binding between ARL13B and Rab35 (Fig. 6D) seems to come from GTP binding to ARL13B rather than Rab35. In addition, ARL13B (G28V) used in Fig. 6E should not work as a GTP-locked mutant. I assume that authors in the previous paper (Dukdulao et al., 2009) tried to make a GTP-locked mutant that corresponds to Kras G12V. Glycine 12 of Kras is located at X2 position of GX1X2X3X4GKS/T motif (G1 loop). G1 loop is GLDNAGKT in human ARL13B, and substitution of invariant glycine to valine should lower affinity of ARL13B for GDP/GTP, rather than locking it at the GTP-bound state. Authors should simply perform a co-IP experiment with the use of GDP-locked (S22N) and GTP-locked (Q67L) mutant of Rab35 to test the hypothesis.

We agree with the reviewer's point and removed the data using the nucleotide-binding mutants of Arl13b and the co-IP experiments with GTPγS or GDP. Following the reviewer's suggestion, we attempted the co-IP experiments using the GDP-locked and GTP-locked mutants of Rab35, but due to variations in the expression levels of Rab35 wildtype and mutants proteins as well as the co-expressed ARL13B-FLAG, it was not possible to quantify the differences in their interaction with Arl13b. Instead, to assess whether the nucleotide-bound state affects the interaction, we immunoprecipitated GFP-RAB35 or ARL13B-GFP with anti-GFP beads, and we then exchanged these GTPases with either GTPγS or GDP using experimental conditions previously described for RAB35 (Kobayashi et al. 2015). Importantly, the subsequent incubation with cell lysates demonstrated that the RAB35-ARL13B interaction is greatly enhanced when either protein is GTP-bound (see new Figure 6D and E).

Minor point

1) line 179-181. "We found that RAB35-S22N overexpression exerts a dominant-negative effect on ciliogenesis, resulting in a severe reduction in ciliation (down to 25% of cells) (Fig 3C)" The ciliation phenotype of cells expressing Rab35-S22N is apparently different from that of Rab35 depleted cells (Fig. 2B). The authors should comment how this difference occurs.

There are multiple examples in the literature where overexpression of inactive GDP-bound variants of Rabs (or related GTPases such as Arls and Arfs) give different phenotypes than depletion or knockout. One explanation for this could be that the GDP-bound variant acts as a dominant-negative mutant by trapping crucial interaction partners (e.g. GEFs) and interfering thus with the function of the endogenous protein. A Rab GTPase can be activated by several GEFs and these GEFs in turn act often on multiple Rabs, thus overexpression of the GDP-bound variant can indirectly affect the function of other Rabs and give therefore different phenotypes compared to a loss-of-function model. This is now alluded to in the discussion.

2) line 219-220. "localisation was observed for DENND1A or TBC1D10B (Fig 3G and H, and Appendix Fig S4C and D)." "Fig. 3G and 3H" should be "Fig. 3H and I".

Now corrected.

3) line 246-247. "compared with 1 and 10%, of non-injected and mismatch MO control embryos, respectively." Does this sentence correspond to Fig. S5B? The data for mismatch MO is missing.

We have included the data for the mismatch MO in Figure EV3 and clarified in the text.

4) line 254. "mismatch MO-injected or non-injected embryo controls (Fig 4D and E)."
Data for non-injected control is missing.

We have included the missing data in Figure 4F and G.

5) line 261-263. "We observed that KV cilia length is significantly reduced in embryos overexpressing Rab35-S22N, when compared with the overexpression of Rab35-WT or mCherry alone (Fig 4F-G)" Authors should mention whether cilia are longer in embryos expressing Rab35-Q67L. If there is no difference between control and Q67L, it should be indicated in the figure (Fig. 6G).

Although there is a trend for an increase in cilia length in embryos expressing Rab35-Q67L, the difference is non-significant. This is now indicated in Figure 4I.

6) line 269-270. "its established role in regulating protein levels at the plasma membrane" References for this statement is missing.

See answer above for Reviewer 2 comment about line 270.

7) line 512. "HEK239T" should be "HEK293T".

Now corrected.

8) line 515. "Cilia formation in hTERT-RPE1, IMCD3 or HEK293T" To help readers to reproduce the data, it would be great if authors could describe how many cells were plated in which plate.

We have added the requested information to the Methods section.

9) line 519 "HEK293T cells were transiently transfected with plasmid DNA using the calcium phosphate precipitation method" How many cells were plated? Please describe calcium phosphate precipitation method in a little more detail.

We have added the requested information to the Methods section.

10) line 522, 524, 526, 533. "TransIT-LT1" "Lipofectamine 3000" "Lipofectamine 2000" What amount of the reagent and plasmid DNA (or siRNA) was used for transfection?

We have added the requested information to the Methods section.

11) line 662-669. "HEK293T cells and hTERT-RPE1 cells were lysed at 4{degree sign}C for 20 min - proteins were eluted in Laemmli sample buffer." For co-IP experiments, how many cells were plated in which plate? What concentration of cell lysates were used for co-IP? What is the total volume of co-IP reaction? What amount of beads was used for co-IP?

We have added the requested information to the Methods section.

12) line 669-673. "For immunoprecipitation with GTPγS -and immunoprecipitations was performed for 2 h" Final concentration of GDP/GTPγS is missing. What concentration of cell lysates were used? Immunoprecipitation performed at 4C? No magnesium added in the buffer?

The immunoprecipitation experiments with GTPγS were replaced with pull-down experiments using GFP-RAB35 bound to anti-GFP beads that were preloaded with either GTPγS or GDP. The details for the new experimental procedure are added to the Methods section.

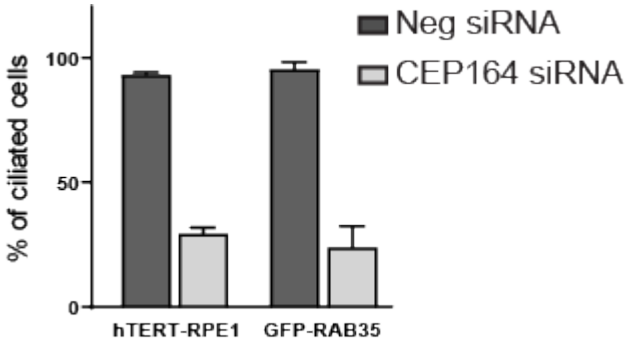
13) Fig. 1C. In line profile plots, each color contains three lines (one is a solid line and the others are dotted lines). Please explain what those three lines are in the figure legend.

The solid line in the line profile plots indicated the mean and the dotted lines the S.E.M values, and we added the explanation to the figure legend.

14) Fig. 2D. Authors generated a clonal cell line expressing GFP-Rab35 for the rescue experiment. Clonal cell line might have different characteristics from the parent cell line. Indeed, cilium length of GFP-Rab35 expressing cells looks shorter than control cells. Similarly, knockdown efficiency might be different between control cells and GFP-Rab35 expressing cells. Authors should show Western blot to confirm that knockdown efficiency is comparable between the two lines. Also, authors should show the expression level of GFP-Rab35 compared with endogenous Rab35.

The GFP-RAB35 expressing hTERT-RPE1 cell line was not derived from a single cell clone, but is a polyclonal cell line with detectable expression of GFP-RAB35 in ~30% of the cells. Due to the low frequency of GFP-RAB35 positive cells in the cell population, it is not feasible to assess RAB35 protein levels by immunoblot in these cells. We agree that it is important to ensure that the siRNA transfection is as efficient in the GFP-RAB35 expressing cells as in the parental hTERT-RPE1 cells. To test this, we included in our knockdown

experiments, as a positive control, an siRNA targeting CEP164, a key ciliogenesis regulator, and confirmed that the cilia loss phenotype in the GFP-RAB35 expressing and parental hTERT-RPE1 cells was identical.



15) Fig. 3A. Did author use polyglutamylated tubulin? Or acetylated tubulin? The leftmost panel was labeled with polyglutamylated tubulin, but the smaller panels were labeled with acetylated tubulin.

We corrected this mistake. Indeed, in Figure 3A, polyglutamylated tubulin was used for all red channel images.

16) Fig. 6A.
A) The data for input and IP is flipped.
B) Authors should mention what percentage of input was used.
C) Ideally, input and IP should be loaded side by side on the same gel, blotted on the same membrane, and detected with the same exposure time. This way allows readers to estimate how strong the interaction is and help them to reproduce the data. Authors should at least mention whether the samples were loaded on the same gel, blotted on the same membrane, and detected with the same exposure time.

This mistake with the inverted immunoblot panels has now been corrected (see new Figure 6A). We added the information regarding the input to the figure legends, and we now show samples loaded on the same gel, blotted on the same membrane and detected with the same exposure time.

17) Fig. 6C. "ARL13B V385A-GFP" should be "ARL13B V358A-GFP".

This is now corrected.

18) Fig. 6D and 6F. Authors should comment why the interaction between Rab35 and ARL13B decreased in the presence of GDP in Fig.6D but increased in Fig. 6F.

This data has been removed.

19) Fig. 7C. Authors should perform Western blot to confirm that knockdown of Rab35 and ARL13B in Rab35/ARL13B double depleted cells was as efficient as either Rab35 or ARL13B single depleted cells. Also, authors should mention that how the double knockdown was performed.

We added the details for the double knockdown experiments to the Methods section. Briefly, hTERT-RPE1 cells were seeded on coverslips and immediately transfected with either Neg or RAB35 siRNA (2 wells for each siRNA). After 24h, a second transfection with Neg or ARL13B siRNA was performed. Thus, all cells were transfected with the same amount of siRNA (Neg/Neg, RAB35/Neg, Neg/ARL13B, RAB35/ARL13B). For readability, we omitted the additional Neg in the labels. As the transfection with the RAB35 siRNA precedes the ARL13B depletion, it is unlikely that there are differences in the knockdown efficiencies between RAB35/Neg and RAB35/ARL13B depleted cells.

20) Table S1. "Human ARL13B (V385A)" should be "Human ARL13B (V358A)"

This is now corrected.

21) Table S2. A catalog number for polyclonal rabbit anti-RAB35 from Proteintech should be "11329-2-AP".

This is now corrected.

2nd Editorial Decision 16 July 2019

Thank you for submitting the revised version of your manuscript. It has now been seen by all of the original referees.

As you can see, all referees find that the study is significantly improved during revision and recommend publication. Before I can accept the manuscript, I need you to address the below minor/editorial points:

REFEREE REPORTS

Referee #1:

All my queries have been answered satisfactorily and the paper can be accepted.

Referee #2:

Kuhns et al., revised

In this revised manuscript, the authors have done a good job of addressing the previous concerns of the reviewers.

The authors have generated RAB35 null cells by Crispr Cas9, providing clear confirmation that RAB35 is a modulator of cilia length in hTERT-RPE1 cells. They have added the quantitative data in the main text and figure legend for the statistic analysis. They added solid data showing the interaction of Rab35 with Arl13B depends on their GTP binding activity.

Most strikingly, the zebrafish images are much improved. The images of long cilia in the KV are of particularly high quality. Because previous work in the zebrafish showed that overexpression of ARL13B leads to long cilia and situs defects, these new data provide good support for their model that RAB35 works together with ARL13B to modulate cilia length.

There are a number of minor points that remain to be addressed:

We previously suggested, "The authors should use a centriole marker or nuclei staining together with acetyl-α-tubulin to quantify the fraction of ciliated cells in the zebrafish experiments (Figure 4)." The authors state in rebuttal letter that they have now quantified the number of cilia per KV but these data do not appear to be in the revised manuscript.

In at least one place, the authors refer to an Appendix supplementary figure, but most of these have been renamed Fig. EV 1-5. Please correct.

It appears that some of the supplemental figures and tables are missing, such as confirmation by quantitative real-time PCR analysis of the efficient depletion of DENND1A, DENND1B, TBC1D10A and TBC1D10B (line 231). Please correct.

Referee #3:

I am convinced. This is a fine paper.

2nd Revision - authors' response 23 July 2019

Referee #2

minor points:

• The authors should use a centriole marker or nuclei staining together with acetyl-α-tubulin to quantify the fraction of ciliated cells in the zebrafish experiments (Figure 4)." The authors state in rebuttal letter that they have now quantified the number of cilia per KV but these data do not appear to be in the revised manuscript.
The cilia number data is described in lines 285-286 of the text and Appendix Figure S3E.

• In at least one place, the authors refer to an Appendix supplementary figure, but most of these have been renamed Fig. EV 1-5. Please correct.
There was one instance where the EV figure was not cited (Pg. 7). This has now been corrected.

• It appears that some of the supplemental figures and tables are missing, such as confirmation by quantitative real-time PCR analysis of the efficient depletion of DENND1A, DENND1B, TBC1D10A and TBC1D10B (line 231). Please correct.
We double checked the supplementary figures and tables, and they are all in the appendix. The real-time PCR data is in Appendix Fig. S2A.

3rd Editorial Decision 31 July 2019

Thank you for submitting your revised manuscript. I have now looked at everything and all looks fine. Therefore I am very pleased to inform you that your manuscript has been accepted for publication in EMBO Reports.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Oliver Blacque
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2018-47625-T.

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- è the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- è figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- è graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- è if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- è Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- è a specification of the experimental system investigated (eg cell line, species name).
- è the assay(s) and method(s) used to carry out the reported observations and measurements
- è an explicit mention of the biological and chemical entity(ies) that are being measured.
- è an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- è the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- è a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- è a statement of how many times the experiment shown was independently replicated in the laboratory.
- è definitions of statistical methods and measures:
  - ↑ common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - ↑ are tests one-sided or two-sided?
  - ↑ are there adjustments for multiple comparisons?
  - ↑ exact statistical test results, e.g., P values = x but not P values < x;
  - ↑ definition of 'center values' as median or average;
  - ↑ definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was determined based on previous publications in the field
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size was always around 30 embryos per experimental condition and per experiment, with n = 3 for all experiments, except if indicated otherwise. To evaluate cilia-related phenotypes, we used 8-10 embryos per condition per experiment (n = 3 independent experiments), with a total of > 300 cilia.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Animals were all scored for organ left-right patterning, and were randomly taken for KV cilia phenotype analysis. Microscope fields of immunofluorescence images were randomly selected for each treatment.
For animal studies, include a statement about randomization even if no randomization was used.	The wildtype animals were randomly crossed, as well as the eggs that were selected for microinjection.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The researchers were not blind when assesing results. The researchers were aware of the fact that subjective bias can influence their results. When possible, automated image analysis with Fiji/ImageJ or CellProfiler was used for image segmentation and quantification to reduce biases.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The researchers were not blind when assesing results. The researchers were aware of the fact that subjective bias can influence their results.
5. For every figure, are statistical tests justified as appropriate?	Yes, the appropriate statistical tests were used and are stated in legend for each figure.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Graphpad Prism software was used for statistical analysis. For data comparing two datasets, Unpaired t test with Welch's correction was used. For data in which more than two datasets were compared, ANOVA followed by Bonferroni post-hoc test or Kruskal-Wallis test (for non-Gaussian populations) followed by Dunn's post-hoc test were used.
Is there an estimate of variation within each group of data?	Yes. Data are presented as mean ± standard error of the mean (S.E.M) or box-and-whisker plots with horizontal lines showing 25, 50 and 75th percentiles and whiskers extending to minimum and maximum values.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The antibodies are listed Appendix Table S2.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293T, NIH3T3, and hTERT-RPE1 cells were regularly tested with Cambrex MycoAlert assay and confirmed to be mycoplasma free IMCD3 were recently adquired at ATCC and are confirmed to be mycoplasma free.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Zebrafish; strains AB background and Tg(β-actin2:loxP-DsRed-loxP-GFP; s928Tg), both genders, and 6-12 months old. To guarantee the welfare of the animals, they were crossed at the most once every 15 days. We used therefore 3 tanks with 10 pairs of progenitors every week. The source of animals was the zebrafish facility of CEDOC/ NMS which is a Techniplast zebrafish-specific aquaria with controlled temperature, pH, salinity, light, and UV-treated water.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The procedures performed in zebrafish were approved by the Portuguese Veterinary Authority (DGAV- Direção Geral de Alimentação e Veterinária) and the welfare of the animals was guaranted.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm compliance

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A