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Cyclic expression of the voltage-gated potassium channel K_V10.1 promotes disassembly of the primary cilium

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Transaction Report:

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

1st Editorial Decision 03 September 2015

Thank you for the submission of your manuscript to our journal. I have taken over its handling as my colleague Barbara is currently not in the office. We have now received the full set of referee reports that is copied below.

As you will see, all referees acknowledge that the findings are potentially interesting and novel. However, they also all point out that the current data are not sufficient to support the hypothesis that Kv10.1-mediated ciliary disassembly impacts on celll proliferation. I think the referees raise valid comments and that all of them should be addressed. Referee 1 mentions in point 8 that some data can be removed from the manuscript, and referee 2 agrees in her/his cross-comments. However, referee 3 feels that data on ciliary localization of Kv10 should be kept, and referee 1 agrees with point 4 of referee 3. Referee 1 also indicates that point 8 of referee 2 does not need to be addressed, which is in agreement with his/her own point 8. It will be important to determine whether Kv10.1 affects cilia formation rather than cilia disassembly. All referees also note that the experiments are not described in sufficient detail and that a number of controls are missing.

From the referee comments it is clear that, as it stands, publication of the manuscript in our journal cannot be considered at this stage. On the other hand, given the potential interest of your findings, I would like to give you the opportunity to address the concerns and would be willing to consider a significantly revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board.

Should you decide to embark on such a revision, acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports

policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further. We would publish your manuscript as a full article, and therefore no shortening is required. However, please keep all materials and methods in the main manuscript file. Regarding the expanded view figures, please upload them as separate files along with the revised manuscript, and please add the figure legends for the expanded view figures at the end of the main manuscript file.

Please change the reference style to the numbered EMBO reports style.

Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The paper describes a previously unanticipated role of Kv10.1 in ciliary disassembly and maintenance. This role is shown in multiple cell types, suggesting that it is non cell type-specific. High expression of Kv10.1 is associated with highly proliferating cells and some cancers. Knockdown of Kv10.1 causes a slower rate of ciliary disassembly, maintenance of cilia in cells entering the S phase and reduced rate of proliferation. Furthermore, gain-of-function mutations in Kv10.1 seem to be responsible with syndromes that could share some phenotypic aspects of known ciliopathies. It is also shown that Kv10.1 is asymmetrically distributed between daughter cells, that might have implications in the proliferation of cancer-initiating stem cells with high levels of Kv10.1. Thus, the paper reports novel and physiologically relevant data. It does fall short though on a mechanism of Kv10.1 action, which is fine at this point, as long as presented data are solid and of high quality. In my view, the paper would substantially benefit from additional experiments to increase the quality of the data, strengthen the evidence that Kv10.1 is indeed responsible for ciliary disassembly in MEF Kv10.1 KO cells using rescue approaches, and provide additional support that block of ciliary disassembly does affect the rate of proliferation. As it stands, the evidence is not strong enough to support the claim that Kv10.1 affects cell proliferation through an effect on ciliary disassembly.

Specific points
1) Fig. 1C and all figures that show representative images of transiently transfected cells, an image should be shown that contains transfected and un-transfected side-by-side. This way, an independent assessment of the effect of the transfected protein could be made. It is unknown if any of the cells shown in Fig. 1C are transfected with Kv10.1. Ideally, GFP should be transfected alone or together with Kv10.1 and quantification of ciliated cells should be shown from GFP-positive cells.

2) Fig. 2A has to be repeated according to point 1 above. It appears that there is an effect on the fraction of ciliated cells and ciliary length upon Kv10.1 knockdown (Fig. 2C and D), but this effects seem to be overcome at later time points. The authors claim that this is consistent with a second wave of ciliation. Which one of the two waves is associated with an effect on cell proliferation?

3) Fig.3 provides strong evidence that Kv10.1 plays a role in disassembly. Wild type and mutant forms should be re-introduced to KO cells and test for effects on ciliogenesis, disassembly, Hedgehog signaling, and cell cycle progression. In fact these cells could substitute NIH 3T3 cells.

4) Fig. 4. The evidence that knockdown of Kv10.1 induces cilia in proliferating cells is indirect. Double staining of cilia and S phase markers should provide more direct evidence that indeed depletion of Kv10.1 induces cilia in cells actually re-entering the cell cycle. As shown in Kim et al, NCB 13:351, 2011, RPE1 cells do not show cilia when they are double-stained with EdU. It'd be interesting to test directly whether this is the case in RPE 1 cells depleted of Kv10.1. This experiment should be accompanied with expression of S phase markers, as was done in the 2011 NCB paper.

5) Fig. 5. Not enough experimental information is given in Fig. 5B to clearly understand what was done in this experiment. This is a point that applies to most of the figures. The authors comment on the results of the figures in the legend, rather than presenting the necessary experimental information, so that the reader can see what was exactly done in this experiment. For example, what cell type was used and what N=12 means. Is it 12 cells in total from one transfection (experiment) or 12 independent transfections? A more appropriate negative control is needed in Fig. 5A. All constructs strongly suppressed ciliation. Are these effects specific? At the very least, another member of the Kv channel family should be used that shows no ciliary expression and lack of reported effects on ciliary disassembly/maintenance.

6) Fig. 6A. These experiments should be done in RPE1 or MEFs. There is no good justification of why a cell types are switched. Specificity of centrosomal/ciliary staining should be shown in Kv10.1 MEF KO cells. Ideally, immune gold staining would help confirm the presence of Kv10.1 in the centrosome/cilium.

7) The authors are encouraged to overexpress in RPE1 or wild type MEFs or reintroduce at least on mutant form of Kv10.1 reported in patients with Zimmermannn-Laband or Temple-Baraitser syndrome in MEF Kv10.1 KO cells and test for effects on ciliation. This relatively simple experiment would significantly enhance the importance of the paper.

8) I find data on ciliary localization signal, association with cortactin, and tumor growth too superficial and not necessary for this manuscript, at this stage. The paper needs to be seriously focused, before going into mechanisms of ciliary targeting, etc.

9) There are early reports showing that Ca2+ influx in response to PDGF BB can induce ciliary disassembly (Tucker et al, Cell 18, 1065, 1979). Can the authors comment on the possibility that Kv10.1 can indirectly affect Ca2+ in this process?

10) There are several typos throughout that need to be taken care of.

Referee #2:

This manuscript by Sánchez et al. describes a novel and significant finding on the role of the voltage-gated potassium channel, Kv10.1, in disassembly of the primary cilium and cell cycle entry/progression in mammalian cell cultures. The authors have performed a number of detailed experiments to show that expression of Kv10.1 induces loss of the cilium and that depletion of the channel either by siRNA knockdown in RPE and NIH3T3 cells as well as in knockout MEFs leads to formation of primary cilia in cycling cells. Further, depletion of the channels leads to overly long cilia as well as increased Hedgehog signaling as judged by increased target gene expression of Gli1. Using fluorescence microscopy analyses with antibodies targeting the endogenous channel and with cell transfected with Kv10.1 constructs, they find that Kv10.1 localizes to the cilia-centrosome axis, supporting the conclusion that Kv10.1 partly exerts its effects on ciliary assembly, maintenance and

disassembly at the primary cilium. These and other findings in their manuscript are exciting and important in the field of primary cilia and tumorigenesis/cancer. On the other hand, as they stand the data are not fully compelling, as outlined below.

1) The conclusions by the authors are largely based on observations obtained in cells serum starved to induce growth arrest and formation of primary cilia - as well as on re-addition of serum to induce ciliary disassembly and cell cycle entry. Since cell-cell contacts in confluent cultures are well-known to induce growth arrest and ciliary formation in different cell types grown in the presence of serum, the authors should include information on cell confluences in their experiments during the various experimental setups.

2) The majority of data are based on siRNA-mediated knockdown of Kv10.1, but only a single western blot is shown (Fig S1) to verify a diminished level of the protein. First of all, it is difficult to read under which conditions the siRNA experiment was conducted. As such, the authors should present western blot data with Kv10.1 and control siRNA for all their experiments to show the level of Kv10.1 is highly reduced for cells grown in the presence of serum, in the absence of serum, and after re-addition of serum to growth-arrested cells to ascertain that changes in ciliary formation, ciliary length and disassembly correlates with a knockdown of the protein. Similarly, the authors must include western blot data to show the expression of Kv10.1 constructs in transfected cells in order to verify expression of the right proteins as well as demonstrating transfection rate. Please also add loading controls for the gels/western blots.

3) The fluorescence microscopy analyses on localization of endogenous as well as constructs to the cilium-centrosome axis are important and critical to the manuscript. The localizations look fine to me, but the authors must verify that e.g. antibody localization is specific by using siRNA to show that the signal disappears (or is greatly diminished) at the cilium. This could be complemented with a western blot of the entire gel, showing that the antibody does not recognize unspecific proteins. Ideally, western blots and immunofluorescence images should be included for KO MEFs to validate antibody specificity. Further, Figure 6B with RPE cells shows localization of anti-Kv10.1 to the cilium as well as to puncta around the ciliary base region after reintroduction of serum for 4 hours. Please explain the reason for this setup, and why immunofluorescence microscopy was not carried out on growth-arrested cells with no reintroduction of serum. Indeed, if Kv10.1 is exerting its effects at the cilia, then it would be interesting to follow its localization also during ciliogenesis, i.e., at different time periods after introduction to serum starvation. The authors show nicely that centrosomal localization of either antibody or venus-tagged Kv10.1 in RPE cells disappears after prolonged (20 h) reintroduction to serum. This should be quantitated and presented as an individual panel in the Figure.

4) Figure 8 shows fluorescence localization of the Kv10.1-venus construct in cells undergoing cytokinesis (upper panel) and what appears to the metaphase of mitosis (lower panels). Based on the panels, the authors suggest that Kv10.1 under these circumstances remains associated with the mother centriole during mitosis. This is a very valid hypothesis, but the data are not compelling and they raise a series of questions: first of all, the legend or main text doesn´t say which cell types were used for the experiment, and secondly, no marker was used for the centrosomes. Indeed the Venus signal in the lower panels points to a localization other than one of the spindle poles, which normally is located in close proximity to the beginning of the spindle (and not about 5 micrometer form this site as depicted in the panels). So centrosomal markers must be included, and if the authors would like to substantiate their idea about localization to the oldest centrosome/mother centriole there are signaling markers available for this, including Smo, PDGFRa and Inversin - please see Anderson and Stearns (Curr Biol. 2009 Sep 15; 19(17): 1498-1502), which also should be included in the manuscript regarding centriole age and asymmetric centrosome localization/function. Thirdly, the results raise the question as to the centrosomal and ciliary localization of cells in cycling cells prior to mitosis and after cytokinesis/cell cycle arrest (see Figures 6 and 7), where there seems to be centrosomal localization in all growth arrested cells + 4h of serum addition (Figure 7A) and no localization at all in cycling cells prior to mitosis (Figure 7B). This needs to be explained.

5) The authors refer to Liem et al. (2012) that increased number of cilia reinforces Shh-induced activation of Hedgehog signaling, and the authors show that expression of the Hh-target gene, Gli2, is upregulated in KO MEFs as compared to wt cells (Figure 3B). However, is difficult to read from the manuscript how this experiment was carried out and whether the authors actually stimulated the

cells with Shh. It is important to know that without ligand (and in cells with defects in formation of primary cilia), Hh signaling can be either upregulated or downregulated depending on the cell type. Further, it is not clear whether the authors used wt MEFs form the same littermate, which is critical for comparing to the KO situation. Preferentially, the experiment should be carried out with control and Kv10.1 siRNA cells and with additional markers, such as Ptch1. Alternatively, immunofluorescence microscopy would show that increased Hh signaling is associated with nuclear localization of FL Gli transcription factors GLi2/3.

6) Please show representative immunofluorescence microscopy data with localization of Ki67 and cilia markers (in Figure 4).

7) Ciliary localization signal (CLS) in Kv10.1: The C-terminal region of Kv10.1 contains a nuclear localization signal, which the authors speculate to play a role in ciliary targeting and function - initially by comparing ciliation frequencies in transfecting cells with vector encoding FL Kv10.1 and a C-terminal-deleted version of Kv10.1 (designated Kv10.1deltaCLS by the authors) (Figure 9C). It is not clear to this reviewer how the experiment was carried. Where the cells first serum-depleted to induce growth arrest and the reintroduced to serum? Please explain and why this setup was chosen. Nevertheless, the data show that expression of the deletion construct does not inhibit ciliogenesis as compared to the FL construct. Further, the authors state from Figure 9D that this was not the case in cells cultured in the continuous presence of serum. Again, this reviewer have difficulties in understanding the meaning of "continous presence". Please explain. Also, a major problem with these experiments is that the authors should not refer to the deletion construct with the term "CLS", since no data are provided suggesting that the C-terminal has a motif, which is either sufficient or required for ciliary localization. The authors should show the aa sequence of the C-terminus (as well as the sequence of the deletion construct) and they should align it with known CLSs to see if there is any resemblance. Further, it is critical that the localization is properly investigated by fluorescence microscopy to validate whether there is a defect in localization to the cilium-centrosome axis. It is also well-known that deletion constructs may attain structural/folding changes that interfere with the ciliary targeting machinery (see e.g. Bhogaraju et al. Cilia 2:10).

8) The data on the role of cortactin are highly interesting and may support the hypothesis by the authors that the function of Kv10.1 in disassembly is regulated via a temporally and restricted crosstalking with cortactin. However, there is no real experimental data to back this up in detail. Further, given the known interaction between Kv10.1 and other proteins that impinge on ciliary assembly/disassembly, it would be important to understand the mechanistic in the ciliary function of Kv10.1 in relation to these proteins - such as through AURA, RabGTPases, etc. Indeed, the immunographs presented in Figure 6B shows that Kv10.1 localizes in puncta around the ciliary base region and similar to what has previously been reported on vesicular trafficking at this site. Did the authors look at co-localization between Kv10.1 and e.g. Rab5, Rab11, Rabaptin, clathrin, etc? The authors may also pay notice to a recent paper on ciliary disassembly from the lab of Dr. Pedersen on the role of PDGF signaling in disassembly, which is associated with AURA and PLCgamma (Nielsen et al. J Cell Sci. 2015 Aug 19. pii: jcs.173559).

9) Page 4, line 1: the authors may consider using more recent reviews on ciliopathies.

10) Page 7, line 19: "whether" is misspelled. Please correct

Conclusions:
Given the current widespread interest in primary cilia, this story will appeal to a wide audience. I recommend strongly publication in EMBO Reports after revision according to the comments in above.

Referee #3:

Disassembly of primary cilium regulates G1-S transition to control cell cycle progression. Overexpression of Kv10.1, a potassium channel, is frequently associated with fast growing tumors. The authors aim to demonstrate whether Kv10.1 regulates ciliary disassembly to exert its function on cell proliferation. This is an interesting problem and an important problem to solve.

I was excited when I read the abstract. However, when I read the ms, I felt I had a lot of problems understanding this ms and could not reach a clear conclusion. The way of presenting the data, the flow of the rational in the text and conclusion reached did not meet my expectations. I have found it very confusing and hard to understand.

Several major problems are listed below though it may not include all the problems. I would encourage the authors to have this ms for a critical reading by some experts in the field.

1). If the authors' conclusion stands, it should include data on cells that are not ciliated. For example, using RNAi to inhibit ciliary formation, and then do a comparison. In this way, you can tell whether Kv10.1 exerts its function on cell cycle is ciliary dependent or independent.

2. It seems to me that Kv10.1 suppresses ciliary formation in quiescent cells, and does not affect ciliary disassembly. In Fig.1, overexpression of Kv10.1 did suppress ciliary formation. However, after inducing ciliary disassembly, the rate of ciliated cells decreases similarly as the control. Second, in Fig. 2, knockdown of Kv10.1 has a similar ciliary length shortening though ciliated cells did not change much.

3. For the cilary localization of Kv10.1, the ciliary localization is less prominent when cells express more Kv10.1 at your experimental conditions (Fig. 6 A and B). Because of this, In Fig. 6A, a co-staining of Kv10.1 and ciliary marker should be used.

4. Potassium channel activity is not required for its ciliary function, but the ciliary targeting sequence at the C-terminus is essential. It is hard to understand when the C-terminus is deleted, it behaves like wild type Kv,10.1. For the ciliary targeting sequence, it needs to demonstrate whether the localization of Kv10.1 in the cilium is affected when this sequence is deleted.

5. In Figure 4, panel B. How can be the number of double positive cells of the control is more than that of single positive cells?

6. Fig. 9, it says that Kv10.1 cilairy targeting sequence is involved in ciliary disassembly. However, related experiments are designed for examination of ciliation.

7. Fig. 5C, it should include data on Kv10.1 knockdown alone, C-terminal overexpression alone.

Minor points,
The authors should carefully check the labeling and spelling. For example, TERT is used for RPE-1 cells (fig 9. C). Fraction ciliated cells should be "fraction of .." for a few Y-axis labelling. Membrane potential should be membrane potential (Fig. 9B). There are also other mis-spellings such as microtubule (p9);

1st Revision - authors' response 31 December 2015

We would like to thank the reviewers for their constructive criticism. Having all comments in mind, it became obvious that there was a general problem in the manuscript regarding clarity and detail in the description of the experiments. This has been taken care of in the new version. Additionally, we have identified two potential sources of problems that we think we have now corrected.

First, we had not stressed sufficiently the fact that expression of KV10.1 is not constant along the cell cycle, and cells in G0 and mid-G1 do not express the channel (see attached manuscript in press). We have modified the title of the manuscript to highlight this fact (now: Cyclic expression of the voltage-gated potassium channel KV10.1 promotes disassembly of the primary cilium). Hence, cells with fully developed cilia typically do *not* express KV10.1.

Conversely, overexpression of KV10.1 eliminates cilia. For these reasons, we can only detect endogenous KV10.1 at the cilium/centrosome, and that only in cilia that are in the process of being retracted.

Second, the actions of KV10.1 are two-fold:

a. Acceleration of ciliary disassembly, which happens during the time window ofexpression of the channel and requires K+ permeation and a CLS.
b. Maintenance of deciliation, which would require extemporaneous expression of the channel (G1/G0), and is independent on transmembrane domains and CLS.

In this new version of the manuscript we have concentrated only on effects on ciliary resorption, because these are more evident in the knockout cells and do not require continued overexpression of the channel, which is non-natural as stated above.

Correcting these issues and addressing the concerns of the referees required extensive rewriting of the manuscript, and therefore we will only detail major changes in the Figures. Figure 5 has been moved now to Fig. EV2 (with some changes), except for panel B which now is Fig. 2E. Fig. 8 has been deleted. Fig. 7 and Fig. 9 have been divided in two; therefore the number of Figures is still 9.

We added new panels in Figures 1, 2, 3, 4, and 9, following requirements of the reviewers. A copy of the manuscript with changes highlighted is also included in the submission. We hope that this new version of the manuscript is now acceptable for publication in EMBO Reports.

Responses to the reviewers.

Referee #1:
1) Fig. 1C and all figures that show representative images of transiently transfected cells, an image should be shown that contains transfected and un-transfected side-by-side. This way, an independent assessment of the effect of the transfected protein could be made. It is unknown if any of the cells shown in Fig. 1C are transfected with Kv10.1. Ideally, GFP should be transfected alone or together with Kv10.1 and quantification of ciliated cells should be shown from GFP-positive cells.

We have now included images of transfected and non-transfected cells side by side. We have however a concern when using only GFP-positive cells to evaluate the overall effect of transfection of KV10.1, because transfected cells expressing small amounts of the proteins would not be detected, and apparently the effect of Kv10.1 requires little amounts to be evident (the endogenous channel is very scarce in all cases). We are aware that looking at a population represents an underestimation of the effect. For GFP-positive cells (See new Figs. 1 and 3) the frequency of presence of cilia is essentially zero.

2) Fig. 2A has to be repeated according to point 1 above. It appears that there is an effect on the fraction of ciliated cells and ciliary length upon Kv10.1 knockdown (Fig. 2C and D), but this effects seem to be overcome at later time points. The authors claim that this is consistent with a second wave of ciliation. Which one of the two waves is associated with an effect on cell proliferation?

As we now more explicitly state in the text, initiation of the cell cycle coincides with a shortening of cilia, but there is afterwards, a re-elongation before the cilia completely disappear (Tucker et al., *Cell* **17**: 527-535, 1979; Spalluto et al., *FEBS Open Bio* **3**: 334-340, 2013). Our observation is that the cilia in KV10.1 knockdown cells behave similarly and do show a re-elongation.

3) Fig.3 provides strong evidence that Kv10.1 plays a role in disassembly. Wild type and mutant forms should be re-introduced to KO cells and test for effects on ciliogenesis, disassembly, Hedgehog signaling, and cell cycle progression. In fact these cells could substitute NIH 3T3 cells.

We have now performed rescue experiments reintroducing KV10.1. Experiments with the mutant form were also performed on both KO and wild type MEFs (the latter are shown in the manuscript). We have maintained 3T3 cells because the effects of KV10.1 knockdown disappeared after two passages and therefore robust statistics was much more difficult to achieve in the primary cells.

4) Fig. 4. The evidence that knockdown of Kv10.1 induces cilia in proliferating cells is indirect. Double staining of cilia and S phase markers should provide more direct evidence that indeed depletion of Kv10.1 induces cilia in cells actually re-entering the cell cycle. As shown in Kim et al, NCB 13:351, 2011, RPE1 cells do not show cilia when they are double-stained with EdU. It'd be interesting to test directly whether this is the case in RPE 1 cells depleted of Kv10.1. This experiment should be accompanied with expression of S phase markers, as was done in the 2011

NCB paper.

We included new data on cells labeled with EdU (Fig. 4D) that confirm our observation of double positive cells upon knockdown of KV10.1. We can reproduce the results in Kim et al. in the wild type cells, but KO cells do show cilia even if EdU positive.

5) Fig. 5. Not enough experimental information is given in Fig. 5B to clearly understand what was done in this experiment. This is a point that applies to most of the figures. The authors comment on the results of the figures in the legend, rather than presenting the necessary experimental information, so that the reader can see what was exactly done in this experiment. For example, what cell type was used and what N=12 means. Is it 12 cells in total from one transfection (experiment) or 12 independent transfections? A more appropriate negative control is needed in Fig. 5A. All constructs strongly suppressed ciliation. Are these effects specific? At the very least, another member of the Kv channel family should be used that shows no ciliary expression and lack of reported effects on ciliary disassembly/maintenance.

Because of the complexity of the effects of KV10.1 overexpression, we have moved most of these experiments to Fig. EV2, except for the illustrative effects in Figure 1. We have completely rewritten the Figure Legends and the text is also simplified and shortened. We have used transfection with KV10.2, the closest relative of KV10.1 to determine if the effects are specific, because both channels are kinetically and structurally very similar. We observed a tendency of KV10.2 to slow down deciliation, thus opposite to KV10.1, nevertheless statistically not significant (Inset in Figure 1D). Importantly, recent evidence shows that KV7.1 channels have the opposite effects to KV10.1 (Slaats et al., *J Cell Sci* 2015, epub ahead of print doi: 10.1242/jcs.176065).

6) Fig. 6A. These experiments should be done in RPE1 or MEFs. There is no good justification of why a cell types are switched. Specificity of centrosomal/ciliary staining should be shown in Kv10.1 MEF KO cells. Ideally, immune gold staining would help confirm the presence of Kv10.1 in the centrosome/cilium.

We chose HFF cells because of the large size of their ciliary pocket. In other cell types, similar experiments stain a smaller area, more difficult to interpret. Our antibodies do not stain any structures in KO MEFs. We are currently optimizing the immune electron microscopy. We do confirm the presence of KV10.1 at the ciliary pocket/ciliary membrane and pericentrosomal vesicles, but the quality of the images (mainly the density of gold particles) is still too low to be shown.

7) The authors are encouraged to overexpress in RPE1 or wild type MEFs or reintroduce at least on mutant form of Kv10.1 reported in patients with Zimmermannn-Laband or Temple-Baraitser syndrome in MEF Kv10.1 KO cells and test for effects on ciliation. This relatively simple experiment would significantly enhance the importance of the paper.

Thank you for this suggestion. We have introduced the mutant in both MEF and RPE1, and the results (for wild type MEFs) are now reported in Figure 3D.

8) I find data on ciliary localization signal, association with cortactin, and tumor growth too superficial and not necessary for this manuscript, at this stage. The paper needs to be seriously focused, before going into mechanisms of ciliary targeting, etc.

We appreciate the opinion of the reviewer, but we have kept part of those data because they reinforce the physiopathological relevance of our observations.

9) There are early reports showing that Ca2+ influx in response to PDGF BB can induce ciliary disassembly (Tucker et al, Cell 18, 1065, 1979). Can the authors comment on the possibility that Kv10.1 can indirectly affect Ca2+ in this process?

We now discuss this possibility.

10) There are several typos throughout that need to be taken care of.

This has been done

Referee #2:
1) The conclusions by the authors are largely based on observations obtained in cells serum starved to induce growth arrest and formation of primary cilia - as well as on re-addition of serum to induce ciliary disassembly and cell cycle entry. Since cell-cell contacts in confluent cultures are well-known to induce growth arrest and ciliary formation in different cell types grown in the presence of serum, the authors should include information on cell confluences in their experiments during the various experimental setups.

We have systematically used non-confluent cultures (at 70-85% confluence), and serum starvation as stimulus for ciliogenesis. This is now clearly indicated in the manuscript.

2) The majority of data are based on siRNA-mediated knockdown of Kv10.1, but only a single western blot is shown (Fig S1) to verify a diminished level of the protein. First of all, it is difficult to read under which conditions the siRNA experiment was conducted. As such, the authors should present western blot data with Kv10.1 and control siRNA for all their experiments to show the level of Kv10.1 is highly reduced for cells grown in the presence of serum, in the absence of serum, and after re-addition of serum to growth-arrested cells to ascertain that changes in ciliary formation, ciliary length and disassembly correlates with a knockdown of the protein. Similarly, the authors must include western blot data to show the expression of Kv10.1 constructs in transfected cells in order to verify expression of the right proteins as well as demonstrating transfection rate. Please also add loading controls for the gels/western blots.

We have included western blots in all conditions tested. Loading controls (Fig. 7B) were not added because the blots correspond to immunoprecipitation, required because of the low abundance of the protein.

3) The fluorescence microscopy analyses on localization of endogenous as well as constructs to the cilium-centrosome axis are important and critical to the manuscript. The localizations look fine to me, but the authors must verify that e.g. antibody localization is specific by using siRNA to show that the signal disappears (or is greatly diminished) at the cilium. This could be complemented with a western blot of the entire gel, showing that the antibody does not recognize unspecific proteins. Ideally, western blots and immunofluorescence images should be included for KO MEFs to validate antibody specificity.

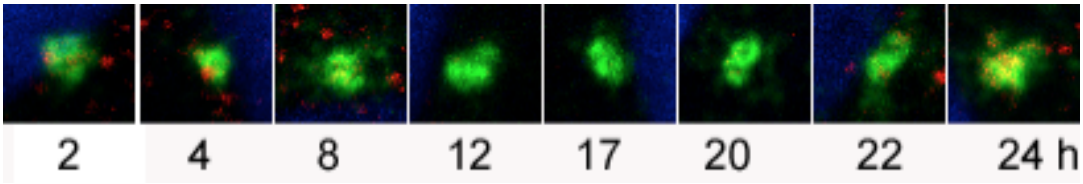
The specificity of the antibody has been thoroughly investigated elsewhere (Hemmerlein et al., *Mol Cancer* 5: 41, 2006), also in KO animals (Ufartes et al., *Hum Mol Genet* **22**: 2247-2262, 2013)

Further, Figure 6B with RPE cells shows localization of anti-Kv10.1 to the cilium as well as to puncta around the ciliary base region after reintroduction of serum for 4 hours. Please explain the reason for this setup, and why immunofluorescence microscopy was not carried out on growth-arrested cells with no reintroduction of serum. Indeed, if Kv10.1 is exerting its effects at the cilia, then it would be interesting to follow its localization also during ciliogenesis, i.e., at different time periods after introduction to serum starvation.

The endogenous expression of KV10.1 is also cell cycle dependent, and minimal (absent) in G0 and S phases; we have included a manuscript in press that documents this in detail. This behavior makes it very difficult to look at the presence of the protein only in ciliated cells (by for example simple serum starvation), because the expression is so low at this point. We are currently constructing a reporter cell line with a labeled KV10.1, but it was not possible to have it on time for the revision of the manuscript. This cell line will be used to follow the localization of the protein along the cell cycle and ciliogenesis, and to do a proper characterization of the asymmetric segregation during mitosis. We have therefore concentrated in the new version on the manuscript only on ciliary resorption.

The authors show nicely that centrosomal localization of either antibody or venus-tagged Kv10.1 in RPE cells disappears after prolonged (20 h) reintroduction to serum. This should be quantitated and presented as an individual panel in the Figure.

We have carefully considered this suggestion, and performed some time course experiments (we include an additional image for appraisal), but due to the oscillation of expression levels we are not sure if the lack of colocalization is due simply to much lower expression, as it appears in the attached image, where there is colocalization with pericentrin at 2 and 4 h, less at 8, and is absent at 12, 17 and 20 h, but appears again at 22 and 24 h, when the expression of KV10.1 rises again. We would like to be careful and limit our reasoning to the re-initiation of the cycle, when we know there is expression of the channel and co-localization with centrosomal proteins as shown biochemically. We therefore removed the data for 20 h.



4) Figure 8 shows fluorescence localization of the Kv10.1-venus construct in cells undergoing cytokinesis (upper panel) and what appears to the metaphase of mitosis (lower panels). Based on the panels, the authors suggest that Kv10.1 under these circumstances remains associated with the mother centriole during mitosis. This is a very valid hypothesis, but the data are not compelling and they raise a series of questions: first of all, the legend or main text doesn´t say which cell types were used for the experiment, and secondly, no marker was used for the centrosomes. Indeed the Venus signal in the lower panels points to a localization other than one of the spindle poles, which normally is located in close proximity to the beginning of the spindle (and not about 5 micrometer form this site as depicted in the panels). So centrosomal markers must be included, and if the authors would like to substantiate their idea about localization to the oldest centrosome/mother centriole there are signaling markers available for this, including Smo, PDGFRa and Inversin – please see Anderson and Stearns (Curr Biol. 2009 Sep 15; 19(17): 1498-1502), which also should be included in the manuscript regarding centriole age and asymmetric centrosome localization/function.

During the remodeling of the paper, after carefully considering the referees' requests and comments, we have decided to dedicate a separate manuscript to the asymmetric segregation studied in detail, and removed the data from the present one.

Thirdly, the results raise the question as to the centrosomal and ciliary localization of cells in cycling cells prior to mitosis and after cytokinesis/cell cycle arrest (see Figures 6 and 7), where there seems to be centrosomal localization in all growth arrested cells + 4h of serum addition (Figure 7A) and no localization at all in cycling cells prior to mitosis (Figure 7B). This needs to be explained.

Again, we attribute this observation to the fact that endogenous KV10.1 is not constantly present and cells in G0/G1 do not express detectable (endogenous) KV10.1. We have explained this better in the new version of the manuscript.

5) The authors refer to Liem et al. (2012) that increased number of cilia reinforces Shhinduced activation of Hedgehog signaling, and the authors show that expression of the Hhtarget gene, Gli2, is upregulated in KO MEFs as compared to wt cells (Figure 3B). However, is difficult to read from the manuscript how this experiment was carried out and whether the authors actually stimulated the cells with Shh. It is important to know that without ligand (and in cells with defects in formation of primary cilia), Hh signaling can be either upregulated or downregulated depending on the cell type.

We have corrected this, and added information obtained from a RT-PCR gene array.

Further, it is not clear whether the authors used wt MEFs form the same littermate, which is critical for comparing to the KO situation. Preferentially, the experiment should be carried out with control and Kv10.1 siRNA cells and with additional markers, such as Ptch1. Alternatively, immunofluorescence microscopy would show that increased Hh signaling is associated with nuclear localization of FL Gli transcription factors GLi2/3.

MEFs are not from the same littermate. As stated earlier, we have only one or two passages to do the

experiments, and therefore we use homozygous crossings to obtain MEFs to avoid the need of genotyping. Nevertheless, the experiments have been performed in at least three different litters with essentially identical results.

6) Please show representative immunofluorescence microscopy data with localization of Ki67 and cilia markers (in Figure 4).

We have done this.

7) Ciliary localization signal (CLS) in Kv10.1: The C-terminal region of Kv10.1 contains a nuclear localization signal, which the authors speculate to play a role in ciliary targeting and function - initially by comparing ciliation frequencies in transfecting cells with vector encoding FL Kv10.1 and a C-terminal-deleted version of Kv10.1 (designated Kv10.1deltaCLS by the authors) (Figure 9C). It is not clear to this reviewer how the experiment was carried. Where the cells first serum-depleted to induce growth arrest and the reintroduced to serum? Please explain and why this setup was chosen. Nevertheless, the data show that expression of the deletion construct does not inhibit ciliogenesis as compared to the FL construct. Further, the authors state from Figure 9D that this was not the case in cells cultured in the continuous presence of serum. Again, this reviewer have difficulties in understanding the meaning of "continous presence". Please explain. Also, a major problem with these experiments is that the authors should not refer to the deletion construct with the term "CLS", since no data are provided suggesting that the C-terminal has a motif, which is either sufficient or required for ciliary localization. The authors should show the aa sequence of the C-terminus (as well as the sequence of the deletion construct) and they should align it with known CLSs to see if there is any resemblance. Further, it is critical that the localization is properly investigated by fluorescence microscopy to validate whether there is a defect in localization to the cilium-centrosome axis. It is also well-known that deletion constructs may attain structural/folding changes that interfere with the ciliary targeting machinery (see e.g. Bhogaraju et al. Cilia 2:10).

As stated above, we have concentrated on ciliary disassembly in the new version of the manuscript. Therefore, only data on cells that re-enter the cell cycle is now included in the main body. We hope this will improve clarity. We have included the sequences surrounding the putative CLS and the deletion, and referenced the algorithm that predicts recognition by importin α. In immunofluorescence experiments (now possible because KV10.1ΔLS-expressing cells do show cilia), we have not detected any hint of colocalization of KV10.1ΔCLS with cilia

8) The data on the role of cortactin are highly interesting and may support the hypothesis by the authors that the function of Kv10.1 in disassembly is regulated via a temporally and restricted crosstalking with cortactin. However, there is no real experimental data to back this up in detail. Further, given the known interaction between Kv10.1 and other proteins that impinge on ciliary assembly/disassembly, it would be important to understand the mechanistic in the ciliary function of Kv10.1 in relation to these proteins - such as through AURA, RabGTPases, etc. Indeed, the immunographs presented in Figure 6B shows that Kv10.1 localizes in puncta around the ciliary base region and similar to what has previously been reported on vesicular trafficking at this site. Did the authors look at co-localization between Kv10.1 and e.g. Rab5, Rab11, Rabaptin, clathrin, etc? The authors may also pay notice to a recent paper on ciliary disassembly from the lab of Dr. Pedersen on the role of PDGF signaling in disassembly, which is associated with AURA and PLCgamma (Nielsen et al. J Cell Sci. 2015 Aug 19. pii: jcs.173559).

Thank you for these suggestions. Association of KV10.1 with Rab GTPases (Rab5, Rab7, Rab11) and rabaptin 5 has been described elsewhere (Ninkovic et al., FEBS Lett 586: 3077-3084, 2012) We have concentrated on cortactin because oft he clear parallelisms observed between CTTN and KV10.1 regarding ciliogenesis, and because we did not observe other major alterations in KO fibroblasts. To reinforce our point, we now include experiments where the interaction with CTTN has been abolished by deletion of a stretch of amino acids at the C-terminus of KV10.1 as described in Herrmann et al., J Biol Chem, 287: 44151-44163 2012.

.

9) Page 4, line 1: the authors may consider using more recent reviews on ciliopathies.

We have done this

10) Page 7, line 19: "whether" is misspelled. Please correct

Thank you. We have corrected this.

Referee #3:
1). If the authors' conclusion stands, it should include data on cells that are not ciliated. For example, using RNAi to inhibit ciliary formation, and then do a comparison. In this way, you can tell whether Kv10.1 exerts its function on cell cycle is ciliary dependent or independent.
We would not like to risk overinterpreting our results. Over the years, we have extensively studied the impact of KV10.1 on the cell cycle of cells that do not show primary cilia (e.g. HeLa), and we are reasonably certain that the effects of KV10.1 on cell cycle go beyond its effects on ciliogenesis.

2. It seems to me that Kv10.1 suppresses ciliary formation in quiescent cells, and does not affect ciliary disassembly. In Fig.1, overexpression of Kv10.1 did suppress ciliary formation. However, after inducing ciliary disassembly, the rate of ciliated cells decreases similarly as the control. Second, in Fig. 2, knockdown of Kv10.1 has a similar ciliary length shortening though ciliated cells did not change much.

We have changed the explanation of this Figure in the text and the corresponding legend. KV10.1 overexpression precludes cilium formation, but its absence has a dramatic effect on disassembly. Overexpression combines acceleration of disassembly with inhibition of new ciliogenesis. Inhibition of *de novo* ciliogenesis does not depend on potassium permeation, can be observed when only the C-terminus is transfected, and does not require a CLS. The effects on disassembly do require permeation and an intact CLS. The strongest evidence for this two-fold action is that overexpression of the C-terminus alone cannot overcome the disassembly phenotype induced by knockdown of endogenous KV10.1. We have decided to concentrate on the effects on disassembly to get the manuscript more focused, and have migrated all the data on ciliogenesis (except Fig. 1) to the expanded view material to avoid confusion.

3. For the cilary localization of Kv10.1, the ciliary localization is less prominent when cells express more Kv10.1 at your experimental conditions (Fig. 6 A and B). Because of this, In Fig. 6A, a co-staining of Kv10.1 and ciliary marker should be used.

We face the problem that whenever we overexpress KV10.1 there are no cilia, so we have to rely on endogenous expression, and this occurs only after cell cycle re-entry. We have more clearly stated this in the new manuscript.

4. Potassium channel activity is not required for its ciliary function, but the ciliary targeting sequence at the C-terminus is essential. It is hard to understand when the C-terminus is deleted, it behaves like wild type Kv,10.1. For the ciliary targeting sequence, it needs to demonstrate whether the localization of Kv10.1 in the cilium is affected when this sequence is deleted.

Please refer to the response to point #2. KV10.1 is no longer targeted to the cilium when the CLS is removed.

5. In Figure 4, panel B. How can be the number of double positive cells of the control is more than that of single positive cells?

This number refers to ciliated cells that are at the same time Ki67-negative (not to the whole population). Now clarified in the text.

6. Fig. 9, it says that Kv10.1 cilairy targeting sequence is involved in ciliary disassembly. However, related experiments are designed for examination of ciliation.

Again related to point #2, deletion of the CLS affected disassembly (resorption), overexpression had then a much smaller effect (if any), but did not affect the suppression of ciliogenesis in serum starved cells (Fig. EV2)

7. Fig. 5C, it should include data on Kv10.1 knockdown alone, C-terminal overexpression alone.

All this data is now shown in Fig. EV2

Minor points,
The authors should carefully check the labeling and spelling. For example, TERT is used for RPE-1 cells (fig 9. C). Fraction ciliated cells should be "fraction of .." for a few Y-axis labelling. Membrane potential should be membrane potential (Fig. 9B). There are also other misspellings such as microtubule (p9);

We have carefully checked this, thank you.

2nd Editorial Decision 11 February 2016

Many thanks for the submission of your revised study to EMBO reports. First of all I would like to apologize for the unusual amount of time it has taken us to get back to you with a decision, but we have just now received the final report on it. The manuscript was sent back to the original referees and while all referees appreciate that some of their concerns have been addressed, they still feel the need for additional, mostly control, experiments and given that they were, in general, positive about the study I would like to give you the exceptional opportunity to revise your manuscript a second time along the lines indicated in the new set of referee reports. With regard to the data on the ciliary targeting of Kv10.1, I understand that you do not want to remove these data and this is fine. The other concerns of the reviewers have to be addressed, though.

Formally, papers in EMBO reports have to be accepted within 6 months of the initial decision, which in your case would be March 3rd, 2016. We are, of course, still interested in publishing your study after this time-point, but we would need to take the novelty into account if your study can only be accepted after this date. Given that the final version of the manuscript might still need to be evaluated by one of the reviewers, please also outline briefly in a point-by-point manner how you have addressed the remaining referee concerns.

During our routine image analysis, we also noticed that there seem to be some spliced/cropped lanes in figure 7 (in row 3 and 4 of panel A) and as it is our journal's policy in such cases, authors have to present raw data for ALL experiments (i.e. raw western blots etc.) to our editorial office. Please do include these data when submitting your final version. In addition, in cases, where splicing was done, please clearly indicate these events through black or white vertical lines.

I look forward to seeing the final version of the study as soon as possible and remain with kind regards.

REFEREE REPORTS

Referee #1:

This is a revised manuscript reporting a role of Kv10.1 in ciliary disassembly. The manuscript has been improved after revisions, but several issues still remain.

1. I requested that representative images of transiently transfected cells, where transfected and un-transfected cells are shown side-by-side should be presented in all figures in which there is any type of transfection (siRNA or expression plasmid). This is only done in Figures 1, 3, and 6B.

2. Does Kv10.2 go to centrosomes/cilia? Is it expressed at the same amount as Kv10.1? What is its effect on ciliary disassembly? I agree that it shows some specificity, but it is only examined under conditions of serum starvation. Under these conditions, the functionality of Kv10.1 is not needed to suppress cilia formation. So, this experiment supports specificity for the effect of Kv10.1 on the suppression of cilia formation, which is not the focus of the current version of the manuscript.

3. I asked in the first round of review, which wave of ciliation is relevant to cell proliferation. If the second wave of deciliation is relevant to cell proliferation, then the study should be repeated showing that knockdown of Kv10.1 affects the second wave (later time points after serum re-

addition), if the authors want to establish that Kv10.1 affects cell proliferation through an effect on cilia.

4. I could not find the data using the SHH array.

5. Fig. 4A. Percentage of cells in boxed area should be shown.

6. Fig. 5A. Staining of Kv10.1 along the axonemal membrane in serum-starved HFFs, is suggested. Only one cell is shown. How valid is this? Serum re-addition seems to eliminate axonemal staining, although levels of Kv10.1 rise. Further experiments should be done to settle whether Kv10.1 is present in the axonemal membrane and is reduced upon serum-re-addition. These are not difficult experiments. However, I don't understand what the message of this experiment is and how relevant is for the manuscript.

7. Page 9, last line is referring to Fig. 6B, not 6C.

8. Data on CLS and cortactin are premature and superficial and should be removed from the manuscript (I made that point in the first round of review). For example there is not a single image showing that deletion of CLS fails to target Kv10.1 to the centrosomes/cilia. In this regard, does CLS targets Kv10.1 to the centrosome, axonemal membrane, or both? This could be a whole new study.

Referee #2:

The authors have responded satisfactorily to most of my concerns, leaving the manuscript suitable for publication in EMBO Reports after minor revision. My concern still relates to antibody specificity as to the localization of Kv10.1 in HFF and RPE cells shown in e.g. Figure 5, since no control experiments are included. I understand that the antibody was previously used in other studies, where specificity was investigated in tissue sections as well as in CHO cells. However, the problem is that antibody specificity may greatly vary between cell types and species, and it is well-known that specificity varies between histochemical and cytochemical approaches. In my opinion, the authors must include some kind of specificity evidence for the localization depicted in Figure 5.

Referee #3:

The current version of the ms is much improved over the last version.

The conclusion drawn from this work is that the effect of the potassium channel on cell proliferation is likely through regulation of ciliogenesis (the last sentence of the abstract). However, in the response to the question 1 of reviewer3, it states that " the effects of Kv10.1 on cell cycle go beyond its effects on ciliogenesis". It seems this argument is contradictory to the conclusion. I can understand that Kv10.1 may have multiple effects on cell cycle. But this work is trying to prove the link between Kv10.1 mediated ciliogenesis and cell proliferation. I do believe additional data is necessary, see question 1 of the reviewer3 from the frist round review. This requirement is not excessive and the experiment is not hard to perform. Please see the paper from Li et al., NCB 2011, 13: 402-411. The authors have used non-ciliated cells to prove the link between Tctex1 mediated ciliogenesis and the cell cycle.

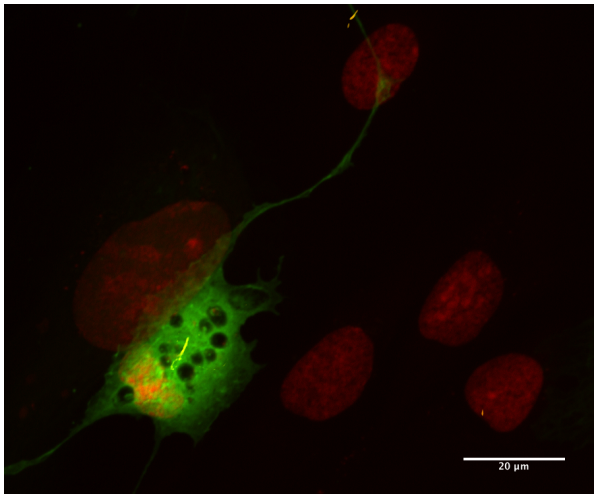
2nd Revision - authors' response 03 March 2016

Referee #1

1. I requested that representative images of transiently transfected cells, where transfected and un-transfected cells are shown side-by-side should be presented in all figures in which there is any type of transfection (siRNA or expression plasmid). This is only done in Figures 1, 3, and 6B.

We respectfully disagree with the referee. We did not include images of siRNA-transfected cells

because the information they give is very limited. We include an exemplary image, but because of the nature of the experiment itself (there will be ciliated cells also in the absence of siRNA), and also because of the lack of unequivocal correlation between green fluorescence and Kv10.1 knockdown, it would be very difficult to draw conclusions from those images.



2. Does Kv10.2 go to centrosomes/cilia? Is it expressed at the same amount as Kv10.1? What is its effect on ciliary disassembly? I agree that it shows some specificity, but it is only examined under conditions of serum starvation. Under these conditions, the functionality of Kv10.1 is not needed to suppress cilia formation. So, this experiment supports specificity for the effect of Kv10.1 on the suppression of cilia formation, which is not the focus of the current version of the manuscript.

We have no evidence of localization of Kv10.2 to centrosomes, but we have no reliable antibodies against this channel. We would like to drive the reviewer's attention to the fact that Kv10.1 virtually abolishes ciliogenesis under the same transfection conditions. We also did not observe any significant effects of Kv10.2 overexpression on deciliation, and actually there was a tendency to slow it down, opposite to Kv101.1. This has now been included in Figure 1. Kv10.2 expression is systematically less prominent than Kv10.1 (as determined in electrophysiological recordings).

3. I asked in the first round of review, which wave of ciliation is relevant to cell proliferation. If the second wave of deciliation is relevant to cell proliferation, then the study should be repeated showing that knockdown of Kv10.1 affects the second wave (later time points after serum re-addition), if the authors want to establish that Kv10.1 affects cell proliferation through an effect on cilia.

Following the reviewer's request, we repeated the time course experiments on longer times (up to 12 hours) and we observed higher frequency of cilia at all time points, as well as in the continuous presence of FCS. Given that (at least in HeLa cells), the expression of Kv10.1 continues to rise after the S phase, it is likely that Kv10.1 participates in both waves. Nevertheless, since we have not been able to test this directly, we have softened our statement.

4. I could not find the data using the SHH array.

This has now been included.

5. Fig. 4A. Percentage of cells in boxed area should be shown.

We have done this.

6. Fig. 5A. Staining of Kv10.1 along the axonemal membrane in serum-starved HFFs, is suggested. Only one cell is shown. How valid is this? Serum re-addition seems to eliminate axonemal staining, although levels of Kv10.1 rise. Further experiments should be done to settle whether Kv10.1 is present in the axonemal membrane and is reduced upon serum-re-addition. These are not difficult experiments. However, I don't understand what the message of this experiment is and how relevant

is for the manuscript.

The cells were serum starved and subsequently stimulated for 4 hours. We apologize for this omission. We have included images of HFF cells stained with Arl13b under the same conditions. The reason for keeping this information in the manuscript is to highlight the fact that Kv10.1 is accessible from the extracellular side, and therefore not only in intracellular vesicles. The large size of the ciliary pocket of HFF cells allows for this kid of experiments.

7. Page 9, last line is referring to Fig. 6B, not 6C.

Thank you for pointing this out, we apologize for the mistake and have corrected it.

8. Data on CLS and cortactin are premature and superficial and should be removed from the manuscript (I made that point in the first round of review). For example there is not a single image showing that deletion of CLS fails to target Kv10.1 to the centrosomes/cilia. In this regard, does CLS targets Kv10.1 to the centrosome, axonemal membrane, or both? This could be a whole new study.

Indeed, we think this is a whole project on its own and deserves further research. As stated in the text, we have found no evidence of ciliary localization of Kv10.1 lacking a CLS.

Referee #2:

The authors have responded satisfactorily to most of my concerns, leaving the manuscript suitable for publication in EMBO Reports after minor revision. My concern still relates to antibody specificity as to the localization of Kv10.1 in HFF and RPE cells shown in e.g. Figure 5, since no control experiments are included. I understand that the antibody was previously used in other studies, where specificity was investigated in tissue sections as well as in CHO cells. However, the problem is that antibody specificity may greatly vary between cell types and species, and it is well-known that specificity varies between histochemical and cytochemical approaches. In my opinion, the authors must include some kind of specificity evidence for the localization depicted in Figure 5.

We have included the requested data. The specificity of the antibody is documented using MEFs from knockout mice (see new Figure 5).

Referee #3:

The current version of the ms is much improved over the last version.

The conclusion drawn from this work is that the effect of the potassium channel on cell proliferation is likely through regulation of ciliogenesis (the last sentence of the abstract). However, in the response to the question 1 of reviewer3, it states that " the effects of Kv10.1 on cell cycle go beyond its effects on ciliogenesis". It seems this argument is contradictory to the conclusion. I can understand that Kv10.1 may have multiple effects on cell cycle. But this work is trying to prove the link between Kv10.1 mediated ciliogenesis and cell proliferation. I do believe additional data is necessary, see question 1 of the reviewer3 from the frist round review. This requirement is not excessive and the experiment is not hard to perform. Please see the paper from Li et al., NCB 2011, 13: 402-411. The authors have used non-ciliated cells to prove the link between Tctex1 mediated ciliogenesis and the cell cycle.

We thank the referee for this suggestion. We think had misunderstood this comment in the previous round. We now provide data obtained in HeLa cells, where knockdown of Kv10.1, which is known to delay the completion of the cell cycle, induces the presence of structures compatible with primary cilia (Fig. EV^

3rd Editorial Decision 09 March 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

REFEREE REPORTS
Referee #1

The authors have addressed my comments and I recommend the manuscript for publication in EMBO Reports.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

Corresponding Author Name: Luis A. Pardo
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2015-41082V1

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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:

- 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.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- 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:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- 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.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- 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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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?	We use powerandsamplesize.com/Calculators when estimating sizes for animal experiments (permit applications), but that was not required in this case.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	From our experience with xenografts of CHO-K1 cells expressing Kv10.1, groups of 6 animals are suffcient to achieve significance. Page 19
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	The animals were not treated, so they were not randomized. Page 19
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.	No, except for measurements of cilium length, which were automated (Page 28)
4.b. For animal studies, include a statement about blinding even if no blinding was done	Only the animal caretaker was blinded.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We did not perform normality tests.
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
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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

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

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).	Antibodies are listed in pages 16-18, with catalog numbers for commercial antibodies and references for non-commercial ones.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	hTERT-RPE1 from ATCC, 3T3 and CHO-K1 from DSMZ, HFF were a gift from S. Christensen (Copenhagen). Page 16. Mycoplasma tests were peridocally performed. hTERT-RPE1 cells were indirectly authenticated through hygromicine resistance.

* 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.	Athymic nude male mice, (NMRI Fox1nu/nu) housed in aseptic conditions. From in-house breeding. Page 18
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Approved by teh State of Lower Saxony. Page 18
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.	Reporting conforms o ARRIVE guidelines

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 accession codes for deposited data. See author guidelines, under '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. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	N/A
22. 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

23. 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.	No