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Rabl2 GTP hydrolysis licenses BBSome-mediated export to fine-tune ciliary signaling

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1st Editorial Decision 9th Jun 2020

Thank you for submitting your manuscript for consideration by the EMBO Journal. We have now received three referee reports on your manuscript, which are included below for your information.

As you will see from the comments, all reviewers appreciate the presented insights into the role of Rabl2 in regulation of ciliary GPCR trafficking. However, they also indicate a number of concerns that should be addressed before they can support publication of the manuscript. Based on the overall interest expressed in the reports, I would like to invite you to submit a revised version of your manuscript, in which you address the comments of all three referees.

I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic, and I would be happy to discuss the revision in more detail via email or phone/videoconferencing.

We have extended our 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. This means that competing manuscripts published during revision period will not negatively impact on our assessment of the conceptual advance presented by your study. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Please feel free to contact me if you have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to receiving your revised manuscript.

Referee #1:

The manuscript by Duan et al makes a compelling case for a previously unappreciated role of Rabl2 as a switch in BBSome-mediated export from cilia. At least three previous studies showed a role of Rabl2 in binding to IFT-B complex in a GTP-dependent manner and in GPCR trafficking into cilia. One of the studies (Kanie et al, 2017) also suggested a role of Rabl2-GTP in triggering anterograde trafficking of IFT-B particles into cilia. Another study showed decreased Gpr161 and Htr6 trafficking to cilia upon Rabl2 RNAi, and correspondingly increased GPCRs upon overexpression (Dateyama et al. 2017). The current study instead uses multiple orthologous approaches, including knockout (ko), knock in mouse models and state-of-the-art cell biological methods including ciliary isolation from mammalian cells, FLIP assays and mutagenesis of GTP-locked forms to convincingly demonstrate the new postulated function of Rabl2 in ciliary export. The study makes multiple major advances in the field as follows.

First, in contrast to previous studies in Rabl2 ko (Kanie et al, 2017) or Rabl2b GDP-locked (Nishijima et al, 2017) RPE cells that had compromised ciliogenesis, the authors convincingly demonstrate ciliogenesis to be unaffected in Rabl2 ko MEFs, kidney tubules, and multicilated cells or upon overexpression of . Rabl2b GDP-locked form in ko MEFs. Chlamydmonas Rabl2 mutants also do not have flagella (Nishijima et al, 2017).

Second, by thoroughly characterizing the Rabl2 ko mice the authors note BBS like phenotypes (polydactyly, obesity, retinal degeneration, male sterility), though some these phenotypes have

been described before in the D73G mutant (Lo et al, 2012; Lo et al, 2016) and partial characterization in Kanie et al, 2017.

Third, the authors revise our current understanding of wild type Rabl2 sublocalization from distal appendages to also include cilia and undergo IFT but excluding the transition zone. The authors use 3NG labeled proteins and SIM for demonstrating these results convincingly.

Four, in contrast to a previous paper that suggested a role of Rabl2 in GPCR trafficking (Dateyama et al, 2019) and another paper (in Bioxriv preprint Barbeito et al, 220) suggesting the same, the authors contend that Gpr161 retrieval from cilia is actually affected in the knockout. The previous effects were shown using RNAi in RPE cells and overexpression. In contrast, the authors use rescue using WT, and GTP mutants of Rabl2 in ko background, and do not see an effect on increased Gpr161 levels from GTP locked form, but rather a lack of retrieval upon SAG treatment. Smo also is enriched in absence of Hh activity, suggesting a lack of removal from cilia, similar to Ift27/BBS mutants.

Five, and most importantly, the authors use an ingenuous method to get pure ciliary preps from multiciliated cells (previous published protocols have cytoplasmic contamination), to confirm binding of Rabl2 in a GTP-dependent manner to IFT-B complex, as seen by others. By using their pure ciliary preps and RPE cells expressing GTP locked form, they next show BBSome, Lztfl1 and Arl6 accumulation in cilia expressing Rabl2 GTP- locked form. Using FLIP experiments, the authors also nicely show delayed exit of BBS5 by the GTP-locked form. They also use another double mutant that likely affect the GTP-locked form in causing export defects.
Overall, the results are robust and of very high quality. By using multiple state-of-the-art technologies in organismal and cell biology assays, the current manuscript performs a detailed and novel characterization of Rabl2 as a GTP switch in retrograde ciliary transport. Although, it is not clear how Rabl2-GTP binding to IFT-B inhibits BBSome exit, which is more of an unanswered question for future study, I have a few comments that might further enhance the presentation of the results to the general reader and strengthen the conclusions of the present manuscript.

1. The authors should at least rule out if they note a reduction of anterograde IFTs in the mouse knockout cilia, as previously reported in Kanie et al, 2017. The lack of cilia in RPE cells could be a consequence of paralogous RABL2 genes in human cells vs a single gene in mouse cells but need to discussed in more detail.

2. As far as I know BBS patients have postaxial polydactyly. The images the authors show in Fig EV1 suggest preaxial polydactly in the sketelal prep (right panel EV1A), but postaxial polydactyly in the left panel (extra 5th digit, not hallux/big toe). The image in Kanie et al, 2017 also shows post-axial polydactyly, which was not mentioned explicitly (Fig 7A). While preaxial polydactyly is caused by increased Shh pathway activity anteriorly in the limb bud, postaxial polydactyly phenotype in BBS patients is not well understood. Considering that both pre- and post-axial polydactyly are seen in Rabl2 knockout, the authors should at least show a skeletal prep that demonstrates post axial polydactyly and also quantify the preponderance of both conditions in the knockout mice. Also, I am curious if they see similar effects in forelimbs. By the way, thumb (legend in Fig EV1) refers to fore limb, not hind limb shown in this figure.

3. The authors need to show and/or quantify data for Rabl2 ko MEFs only for both Gpr161 and Smo levels. Although Smo IF images are shown in Fig 2A, they are not quantified in ko MEFs. Similarly, they should rule out if they see decreased Gpr161 levels in the ko cilia as shown by RNAi in Dateyama et al 2019.

4. It is absolutely necessary to provide % total cellular inputs used in immunoblots for getting an idea of enrichment of ciliary proteins in their ciliary preps (eg 4E, 5C).

5. The effect of the single mutant D73G should also be included for clarity, especially as they mention in discussion of this mutant phenocopying obesity and male infertility (Lo et al, 2012; Lo et al, 2016) as seen in the knockout.

6. The authors generate a knock in for the GTP-locked form of Rabl2 and notice neonatal lethality and duplex kidney. The authors suggest that these knock in mice phencopy Ift27 knockout, but even if this assumption is true, in no way does this suggest that the GTP-locked form is actually following the same pathway as Ift27. The authors need to perform more characterization of the GTP-locked MEFs and demonstrate retrieval defects (although shown in the context of the knockout-lentiviral rescue in Fig. 3). Also, other IFT27 phenotypes, including neural tube patterning and/or polydactyly should be commented on, if possible, for completeness. Also note that Ift27 ko limbs show preaxial polydactyly. As such, the knock in mice data might be reserved for a later and more complete study and is not necessary to bolster either Fig 5 or Fig 6 data showing a role of Rabl2 in BBSome mediated export.

7. The quantification in FIG. 2 has been suggested to show increased GliFL to GliR ratio in ko. However, the immunoblot shown in 2F and 3H barely looks to have any difference. For further clarity Gli3-FL and Gli3R quantification can be shown separately in addition to the ratios. Please also note that GliFL has often been mentioned as GliA in the text, which is not always correct. Embryo immunoblots should also be shown, instead of quantification alone. Do the authors also see a decrease of Gli1/Ptch1 transcript levels in the knockout E10.5 embryos? Individual data points for different experiments should be shown as the variances in knockout MEFs in Fig. 2G are huge. Fig 3H data has not been quantified, unless there is no difference in Gli3R processing as implied in Fig 2. To me, the causes of decreased Gli1 levels in Rabl2 knockout remain unexplained. If such reduction at all happens, it could also be reflected during neural tube development, which has not been tested in the knockout embryos. As mentioned before in comment 2, preaxial polydactyly as shown in Fig EV1 skeletal preps is a sign of increased Hh signaling, which could result from decreased GliR formation, even though targets such as Gli1 is not upregulated (eg see PMID: 15930098).

8. The discussion should clearly discuss disparities with other papers and underlying reasons for such differences. I tried to point to some of these disparities. Alternatively, the authors might propose a role of Rabl2 in both anterograde and retrograde trafficking clearly (they do mention this in the beginning of the Discussion, but as mentioned in comments 1 and 3, need to be tested for further clarification).

9. Appendix: please mention source of IFT140 ab, Typos: Guinea Pig.

10. Please clearly mention lentiviral infection and selection protocols (how many days, how many passages of MEFs used etc.) in ko MEFs in methods.

Referee #2:

Rabl2 GTP hydrolysis licenses BBSome-mediated export to fine-tune ciliary signaling by Duan et al.

In this dense study, the authors analyze the role of the small GTPase Rabl2 in ciliary traffic, particularly the transport of hedgehog proteins. Using a Rabl2 knock-out mouse, they show that loss of the protein does not interfere with the assembly of cilia. However, the SAG-induced re-localization of hedgehog (Hh) signaling proteins (i.e., the import of Smo, export of GRP161 and the tip accumulation of Gli) is defective in mutant MEFs and transfected RPE1 cells. This phenotype, normal ciliogenesis but impaired movements of hedgehog proteins, is typical for Bardet-Biedl syndrome (BBS) mutants and certain intraflagellar transport mutants (e.g., IFT25/27). A similar phenotype is caused by overexpression of Q80L, a GTP-locked version of Rabl2 but not S35N, a putative GDP-locked derivative. Then, the work explores how Rabl2 ensures the export of Hh proteins from cilia using an impressive combination of mass spec of isolated cilia, life imaging, density gradient centrifugation, generation of a Q80L knock-in mouse, IPs etc.. The authors conclude that GTP hydrolysis of Rabl2 licenses the BBSome and its associated cargoes to pass through the transition zone, a putative gate at the ciliary base, and exit the cilium.
This is a remarkable study, which re-defines and expands our understanding of Rabl2's role in ciliary protein transport. The usage of mouse models shows that Rabl2 is not required for general IFT, as previously reported, and that it is needed for Hh signaling and the export of Hh proteins from cilia. It also demonstrates that tagged "wild-type" Rabl2 undergoes IFT. The effects of the Q80L and other mutations are meticulously characterized. As I will outline below, I have some remaining doubts on the validity of the molecular model that proposes that the GTP cycle of Rabl2 regulates BBSome transit through the transition zone. I think this could be addressed by some rewriting and moderation of the conclusions.

Main point
The key statement of the article is
"(Rabl2's) GTP hydrolysis enables the outward TZ passage of cargo-loaded BBSomes with retrograde IFT machinery, whereas its persistent association leads to their shedding from IFT-B during the passing process and consequently ciliary retention."
This would provide a mechanism for the recently proposed that the concept that the transit of (loaded) BBSomes through the TZ is a catalyzed event (Ye et al. 2018). Ye et al. observed only three such exit events over a 21 hour period. Here, the authors propose a critical role of Rabl2 GTP-cycle in catalyzing the exit. However, the actual exit events or the shedding of BBSomes from IFT were not observed directly in this study. Thus, I think the statement above is not definitive and other explanations are possible.
The supporting evidence is largely based on overexpression of the GTP-locked Q80L construct, which stably binds to IFT, causes an accumulation of BBS proteins in cilia etc. In such cells, the release from tagged BBS3 from cilia is decreased but IFT of IFT27GFP and BBS5-3xNG still occurs suggesting that it is specifically the exit step at the base of cilia, which is impaired. However, only the velocity of IFT was determined and it remains unclear whether the frequency of IFT and BBS transport are affected or not. Also, the measurements of FLIP bleaching experiments are spread-out widely and I couldn't flow how the data were normalized and corrected for the loss of fluorescence especially considering that BBS proteins are enriched 8-12x in Q80L cilia. If BBS5-3xNG is also significantly enriched in cilia, a longer half-life of the signal does not necessarily mean that less BBS5 particles exited the cilium. Actually, the opposite might be true.
Also, the GTP-locked Q80L form could have non-physiological negative effects, such as sequestering of GEFs, which has been described for other GTP-locked GTPases. The key question remains whether it is the physiological role of Rabl2 to permit BBSome exit from cilia. Most features of BBS mutants (polydactyly, kidney anomalies etc.) are also observed in hypomorphic IFT mutants. So, it is possible that loss of Rabl2 interferes with IFT and only indirectly affects BBSome traffic because the latter requires well-tune IFT. Indeed the phenotype of the Q80L mutation and knock-

out are strikingly similar to Ift27-/-, as noted by the authors. So, I wonder if Rabl2 Q80L actually interacts with IFT25/27 blocking their ability to export BBSomes from cilia. To my knowledge, it is unclear if BBSome traffic by IFT is indeed impaired in the ift27 mutants. So it is unclear to me how loss of IFT27 is distinct from Rabl2Q80L. Instead of generally impairing IFT transport of BBSomes such mutations could prevent cargo-bound active BBSomes from binding to its IFT docking site. Finally, the hedgehog defects of Bbs null and Rabl2 mutant animals are comparatively mild when compared to, e.g., some of the IFT-A and -B mutants. Thus, it is unclear whether the strong defects in the distribution of hedgehog proteins observed in ift27, Rabl2 and the Q80L mutant cells are similarly occurring in the corresponding animal models. All in all it seems that loss of Rabl2 and the Q80L allele are simply causing a minor defect in retrograde IFT with the known consequences on BBSome traffic and Hh signaling.
All this said, this is an excellent work firmly establishing a connection between Rabl2 and Hh signaling, the BBSome and retrograde IFT. The authors could address this concern in the discussion section developing their conclusion or hypothesis step-by-step and provide the pro and contra arguments, as necessary.

Minor points
Repeatedly, it is stated that the exit process applies to "loaded BBSomes". Is that meant to exclude a role of Rabl2 in the exit of unloaded BBSomes?

"To understand how Rabl2-GTP blocks the BBSome passage through TZ, we screened for a Rabl2Q80L-based mutant that entered cilia without causing BBSome accumulation and identified the Rabl2D73GQ80L double mutant."
As mentioned in the discussion, a Rabl2-D73G mutant was previously described by Lo et al.. If this work inspired generation of this specific double mutant, it should be cited in the results as well.

p16, bottom: exit should be exist?

Fig. 5G) Since no 3D-SIM of control cilia is provided, I am not sure about the point of this figure.

Fig. 5H) in the second graph, the legend for the X-axis has moved.

Referee #3:

This manuscript by Dr. Xueliang Zhu's lab reports the essential function of a small GTPase Rabl2 for fine-tuning cilia-dependent shh signaling, which is critical normal embryonic development and organismic homeostasis. By rigorous and careful studies of GTP-locked and GDP-locked mouse Rabl2 mutants, the authors reveal that the GTPase activity of Rabl2 controls the proper retrieval of ciliary GPCRs involved in shh signaling. Although there are some concerns remaining in this study as listed below, overall teh major conclusion of this manuscript is very interesting. the manuscript itself is well-written, with robust and clear data. My major comments are listed below:

1. Current studies suggest a two-diffusion-barriers model for the ciliary exit of signaling receptors, the transition fiber (TF) passage and transition zone (TZ) passage. A detailed single molecule imaging study revealed that more than 99% of activated GPCR that have crossed the TZ by BBSome train will bounce back to the ciliary compartment, likely dammed by the periciliary barrier (TF) (Fan Ye et al., 2018). RABL2 has been shown to colocalize with or very proximate (Inner PCM) to transition fibers. Knockdown of TF components compromised the ciliary base localization of

RABL2 (Dateyama et al, 2019; Kanie et al.,2017), indicative of the association or dependence of RABL2 with TFs. The 3D-SIM imaging data (Figure 5G) also shows that GFP-Q80L actually can pass through the TZ and probably reach to TFs. Therefore, whether Rabl2 regulates the ciliary exit of GPCRs by controlling the BBsome mediated TZ passage or TF passage need to be further determined by super-resolution imaging. Specfically, Does GPCRs (e.g. Gpr161) can reach to the intermediate compartment between the TZ and TFs in Rabl2-Q80L-expressing cells?

2. The authors show that Rabl2-Q80L does not affect ciliary abundance of IFT components. However, this does not necessarily mean that IFT function is not affected. The velocity and processivity of IFT machinery in Rabl2-Q80L and proper control cells should be examined.

3. The conclusion that "Rabl2-GTP enters cilia through IFT" is overstated (Figure 4). Indeed, the GTP-bound Rabl2, but not GDP-bound form, has strong affinity to IFT-B complex. Direct evidence needed to show that disrupted IFT would affect the cilia entry of Rabl2. The authors should also consider how to reconcile with the previous study that GTP-RABL2B triggers cilia import of IFT-B (Kanie et al., 2017). Knocking down may IFT-B components produce truncated cilia, which could be used for the experiment.

4. Given the functional redundancy of human RABL2A and RABL2B and its evolutional conservation, it is quite surprising that murine Rabl2 is dispensable for ciliogenesis. Is there any possibility that a redundant player for Rabl2 exists in mouse cells? Although the net ciliation ratio was not affected, to comprehensively understand the functional role of Rabl2 in ciliogenesis, cilia stability and cilia length in Rabl2-/- cells need to be carefully examined.

5. As Rabl2Q80LD73G markedly reduces its association with IFT-B, and IFT-B is essential for ciliary entry of Rabl2 as claimed by the authors, it is quite surprising that the Rabl2Q80LD73G mutant still enters the cilium and show strong anterograde and retrograde IFT (Figure 6). What is the explanation?

Response to reviewers' comment:

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The manuscript by Duan et al makes a compelling case for a previously unappreciated role of Rabl2 as a switch in BBSome-mediated export from cilia. At least three previous studies showed a role of Rabl2 in binding to IFT-B complex in a GTP-dependent manner and in GPCR trafficking into cilia. One of the studies (Kanie et al, 2017) also suggested a role of Rabl2-GTP in triggering anterograde trafficking of IFT-B particles into cilia. Another study showed decreased Gpr161 and Htr6 trafficking to cilia upon Rabl2 RNAi, and correspondingly increased GPCRs upon overexpression (Dateyama et al. 2017). The current study instead uses multiple orthologous approaches, including knockout (ko), knock in mouse models and state-of-the-art cell biological methods including ciliary isolation from mammalian cells, FLIP assays and mutagenesis of GTP-locked forms to convincingly demonstrate the new postulated function of Rabl2 in ciliary export. The study makes multiple major advances in the field as follows.

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Third, the authors revise our current understanding of wild type Rabl2 sublocalization from distal appendages to also include cilia and undergo IFT but excluding the transition zone. The authors use 3NG labeled proteins and SIM for demonstrating these results convincingly.

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using their pure ciliary preps and RPE cells expressing GTP locked form, they next show BBSome, Lztfl1 and Arl6 accumulation in cilia expressing Rabl2 GTP- locked form. Using FLIP experiments, the authors also nicely show delayed exit of BBS5 by the GTP-locked form. They also use another double mutant that likely affect the GTP-locked form in causing export defects.

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Response:

We thank our reviewer for recognizing the strength and implications of the study. The summaries of our reviewer have significantly helped us in revising the discussion section.

1. The authors should at least rule out if they note a reduction of anterograde IFTs in the mouse knockout cilia, as previously reported in Kanie et al, 2017. The lack of cilia in RPE cells could be a consequence of paralogous RABL2 genes in human cells vs a single gene in mouse cells but need to discussed in more detail.

Response:

We appreciate the comments. During the revision, we measured ciliary length and observed only a slight reduction in the *Rabl2*-deficient MEFs (2.3 $\pm$ 0.6 μm vs. 2.5 $\pm$ 0.7 μm in wt MEFs) (Fig 1J). We quantified the ciliary intensity of Ift81 in MEFs and failed to observe a significant difference between the ko and wt groups (Fig 1L). We also analyzed ciliary IFT-B behaviors in live cells and did not find striking differences between groups expressing exogenous Rabl2 and Rabl2^{Q80L} (Fig 5I). These results strengthen the idea that Rabl2 is largely dispensable for ciliary entry of IFT-B.

Following the request by our reviewer, we have further discussed the difference between the human and mouse orthologues in the revised manuscript.

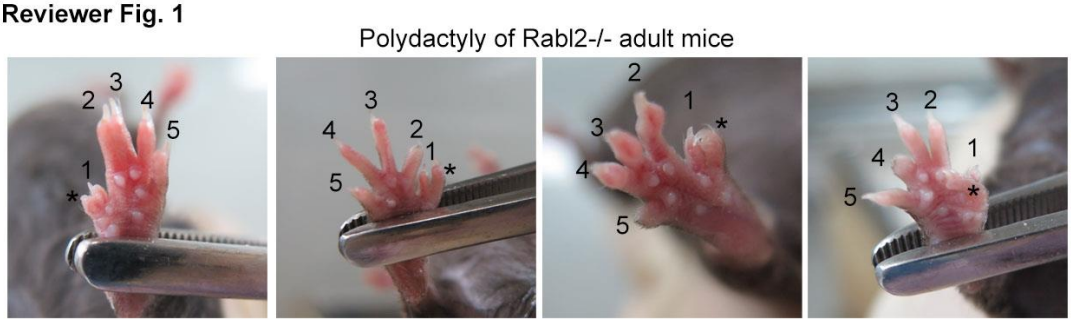
2. As far as I know BBS patients have postaxial polydactyly. The images the authors show in Fig EV1 suggest preaxial polydactly in the sketelal prep (right panel EV1A), but postaxial polydactyly in the left panel (extra 5th digit, not hallux/big toe). The image in Kanie et al, 2017 also shows post-axial polydactyly, which was not mentioned explicitly (Fig 7A). While preaxial polydactyly is caused by increased Shh pathway activity anteriorly in the limb bud, postaxial polydactyly phenotype in BBS patients is not well understood. Considering that both pre- and post-axial polydactyly are seen in Rabl2 knockout, the authors should at least show a skeletal prep that demonstrates post axial polydactyly and also quantify the preponderance of both conditions in the knockout mice. Also, I am curious if they see similar effects in forelimbs. By the way, thumb (legend in Fig EV1) refers to fore limb, not hind limb shown in this figure.

Response:

We are sorry for the confusion. We only observed preaxial duplication of digit 1 in the hind limbs. Of the 151 *Rabl2*-deficient mice that we examined (Fig EV1), 62 displayed the preaxial duplication at one side and 24 at both sides (Fig EV1A,B). To avoid confusion, we have included labels on different digits in Figure EV1A and the numbers of affected mice in the figure legend. We have also replaced "thumb" with "digit 1" in the revised manuscript.

Shown below (**Reviewer Fig. 1**) are hind limbs of four adult mice with polydactyly, in which the preaxial duplication phenotype can be easily visualized.

Reviewer Fig. 1



3. The authors need to show and/or quantify data for Rabl2 ko MEFs only for both Gpr161 and Smo levels. Although Smo IF images are shown in Fig 2A, they are not quantified in ko MEFs. Similarly, they should rule out if they see decreased Gpr161 levels in the ko cilia as shown by RNAi in Dateyama et al 2019.

Response:

As requested, we quantified the IF intensity of Smo and have presented the results in Figure 2A. Statistical test did not reveal a significant difference between the wt and ko populations (Fig 2A). Unfortunately, as the anti-GPR161 antibody (Proteintech, 13398-1-AP) does not recognize mouse Gpr161, we are unable to provide the results for Gpr161. We hope that our reviewer would agree that this piece of data is not essential to our current manuscript.

4. It is absolutely necessary to provide % total cellular inputs used in immunoblots for getting an idea of enrichment of ciliary proteins in their ciliary preps (eg 4E, 5C).

Response:

1/400 of the mEPC lysates and 1/20 of the cilia lysates were loaded per lane in these experiments. We have included a similar description in the legends of Figure 4E and 5C and provided detailed information in the Method section in the revised manuscript.

5. The effect of the single mutant D73G should also be included for clarity, especially as they mention in discussion of this mutant phenocopying obesity and male infertility (Lo et al, 2012; Lo et al, 2016) as seen in the knockout.

Response:

We examined the D73G mutant as requested and have presented the results in Fig EV4.

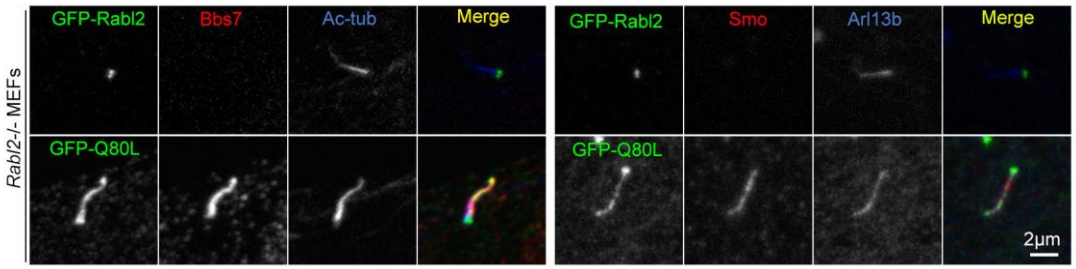
GFP-Rabl2^{D73G} localized to the basal body in RPE1 cells (Fig EV4A), similar to wild-type Rabl2 (Fig 1) and RABL2B^{D73G}-GFP (Nishijima et al., 2017). Nevertheless, unlike RABL2B^{D73G}-GFP (Nishijima et al., 2017), GFP-Rabl2^{D73G} did not inhibit ciliogenesis (Fig EV4A). It also did not induce ciliary accumulation of BBSome and SMO or affect the ciliary translocation of SMO in response to SAG treatment (Fig EV4B,C).

6. The authors generate a knock in for the GTP-locked form of Rabl2 and notice neonatal lethality and duplex kidney. The authors suggest that these knock in mice phencopy Ift27 knockout, but even if this assumption is true, in no way does this suggest that the GTP-locked form is actually following the same pathway as Ift27. The authors need to perform more characterization of the GTP-locked MEFs and demonstrate retrieval defects (although shown in the context of the knockout-lentiviral rescue in Fig. 3). Also, other IFT27 phenotypes, including neural tube patterning and/or polydactyly should be commented on, if possible, for completeness. Also note that Ift27 ko limbs show preaxial polydactyly. As such, the knock in mice data might be reserved for a later and more complete study and is not necessary to bolster either Fig 5 or Fig 6 data showing a role of Rabl2 in BBSome mediated export.

Response:

We observed similar retrieval defects in *Rab2*-defecient MEFs expressing GFP-Rabl2^{Q80L} in the rescue experiments, as shown in the Reviewer Figure 2 below:

Reviewer Fig. 2



In addition to the retrieval defects in RPE1 cells expressing GFP-Rabl2^{Q80L} (Fig 3E,F, and 5), we have presented results showing Bbs5 accumulation in both multicilia and primary cilia of *Rabl2*^{Q80L} KI/KI ependymal cells (Fig 7F and G) and Smo accumulation in the absence of SAG stimulation in the *Rabl2*^{Q80L} KI/KI primary cilia (Fig 7G). In the results presented in Figure 7F and G, the retrieval defects were caused by endogenous Rabl2^{Q80L}. We therefore choose not to present the redundant results in the MEFs. We agree with our reviewer that current results do not sufficiently suggest that the GTP-locked form of Rabl2 is following the same pathway as Ift27 and have toned down the wording in Discussion by changing "indicate" to "appear to indicate".

Following the request of our reviewer, we have mentioned abnormal neural tube patterning and preaxial polydactyly of *Ift27*-deficient mice in the Discussion. We also thank our reviewer for allowing us to leave the detailed characterization of the KI mice to the future.

7. The quantification in FIG. 2 has been suggested to show increased GliFL to GliR ratio in ko. However, the immunoblot shown in 2F and 3H barely looks to have any difference. For further clarity Gli3-FL and Gli3R quantification can be shown separately in addition to the ratios. Please also note that GliFL has often been mentioned as GliA in the text, which is not always correct. Embryo immunoblots should also be shown, instead of quantification alone. Do the authors also see a decrease of Gli1/Ptch1 transcript levels in the knockout E10.5 embryos? Individual data points for different experiments should be shown as the variances in knockout MEFs in Fig. 2G are huge. Fig 3H data has not been quantified, unless there is no difference in Gli3R processing as implied in Fig 2. To me, the causes of decreased Gli1 levels in Rabl2 knockout remain unexplained. If such reduction at all happens, it could also be reflected during neural tube development, which has not been tested in the knockout embryos. As mentioned before in comment 2, preaxial polydactyly as shown in Fig EV1 skeletal preps is a sign of increased Hh signaling, which could result from decreased GliR formation, even though targets such as Gli1 is not upregulated (eg see PMID: 15930098).

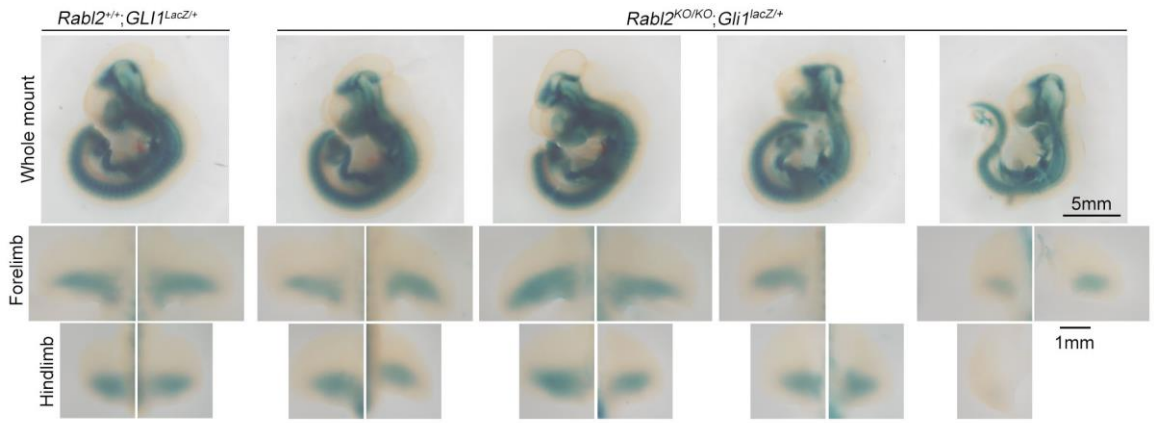
Response:

We thank our reviewer for the comments. As requested, we have included the embryo immunoblots and presented the Gli3-FL and Gli3-R band intensities separately in addition to ratios in Figure 2D and E in the revised manuscript. We have also modified the text to avoid referring Gli-FL as Gli-A. In addition, data points have been included in all our quantification results in the manuscript for better clarity.

Mammalian Hedgehog pathway is indeed very complicated. The final outcome is affected not only by levels of key components, such as Ptch1 and different Gli-A and Gli-R, but by their spatiotemporal distributions in tissues as well. We readily observed decreased ciliary-tip Gli2 (Fig 2B, 3G) and reduced Gli1 levels (Fig 2C; Fig 3H, lanes 1 &2 vs. lanes 3&4) in *Rabl2*-deficient MEFs upon SAG stimulation. Situations in developing embryos, however, appear to be more intricate when we examined *Gli1* transcripts indirectly through X-gal staining. We removed the *LacZ* and *Neo* genes from the *Rabl2*$^{-}$ allele (Fig 1C) by crossing with *Flp* mice (Takeuchi et al., 2002) and then crossed the resultant mice (the resultant *Rabl2* allele is herein named *Rabl2*^{KO} to distinguish from *Rabl2*$^{-}$) with *Gli1*^{lacZ} mice (Bai et al., 2002). Comparing to E10.5 *Rabl2*$^{+/+}$;*Gli1*$^{lacZ/+}$ embryos, *E10.5 Rabl2*$^{KO/KO}$;*Gli1*$^{lacZ/+}$ embryos still displayed substantial X-gal signals (please refer to **Reviewer Fig. 3**), though some *Rabl2*$^{KO/KO}$ embryos (e.g., the two to the right) appeared to show reduced X-gal staining. Variations in shape, area, and intensity of X-gal staining were also observed at limb buds of the *Rabl2*$^{KO/KO}$ embryos (**Reviewer Fig. 3**). The substantial X-gal staining is not unexpected because the viable *Rabl2*-deficient mice with medium penetrance (57%) of polydactyly (Fig EV1) do not suggest a very strong influence on hedgehog signaling. Nonetheless, we feel that these X-gal results are still preliminary for publication. We still need more litters and more detailed examinations on other tissues and find proper ways to quantify the variations. We also need to clarify whether variations were due to delayed development of the embryos (such as the rightmost embryo in **Reviewer Fig. 3**). Embryos at later developmental stages are also required to clearly attribute certain changes to phenotypes such as polydactyly. As detailed effect of *Rabl2* deficiency on the hedgehop signaling is not the major focus of this manuscript, we choose not to present these premature results and leave the detailed

investigations to future research. Furthermore, as our reviewer has pointed out, polydactyly can occur without changes in *Gli1* expression. In this context, the reduced Gli3-R production (Fig 2D) would likely be more relevant to the *Rabl2* deficiency-induced polydactyly.

Reviewer Fig. 3



Following our reviewer's suggestion, we also quantified Gli3 FL/R band intensities from Figure 3H and two additional sets of immunoblots, each set using MEFs prepared from different Rabl2-deficient embryos (Figure 3H). Quantification suggested that, in the absence of SAG, Gli3 FL/R ratios increased in the GFP groups as compared to the wild-type MEFs or the GFP-Rabl2 groups (Fig 3H). After SAG treatment, however, only the reduction between the GFP-Rabl2 and the GFP-Rabl2^{Q80L} groups was statistically significant (Fig 3H). We have also included the immunoblot probed with anti-Rabl2 antibody (Fig 3H). Due to the marked level difference between exogenous and endogenous Rabl2, the wild-type groups were only used to compare with the GFP groups in statistical analyses (Fig 3H).

8. The discussion should clearly discuss disparities with other papers and underlying reasons for such differences. I tried to point to some of these disparities. Alternatively, the authors might propose a role of Rabl2 in both anterograde and retrograde trafficking clearly (they do mention this in the beginning of the Discussion, but as mentioned in comments 1 and 3, need to be tested for further clarification).

Response:

We thank our reviewer for the instruction. We have extensively modified the discussion section as suggested.

9. Appendix: please mention source of IFT140 ab, Typos: Guinea Pig.

Response:

We have added the information in the revised Table S1.

10. Please clearly mention lentiviral infection and selection protocols (how many days, how many passages of MEFs used etc.) in ko MEFs in methods.

Response:

We have included the requested information in the revised manuscript.

Referee #2:

Rabl2 GTP hydrolysis licenses BBSome-mediated export to fine-tune ciliary signaling by Duan et al.

In this dense study, the authors analyze the role of the small GTPase Rabl2 in ciliary traffic, particularly the transport of hedgehog proteins. Using a Rabl2 knock-out mouse, they show that loss of the protein does not interfere with the assembly of cilia. However, the SAG-induced re-localization of hedgehog (Hh) signaling proteins (i.e., the import of Smo, export of GRP161 and the tip accumulation of Gli) is defective in mutant MEFs and transfected RPE1 cells. This phenotype, normal ciliogenesis but impaired movements of hedgehog proteins, is typical for Bardet-Biedl syndrome (BBS) mutants and certain intraflagellar transport mutants (e.g., IFT25/27). A similar phenotype is caused by overexpression of Q80L, a GTP-locked version of Rabl2 but not S35N, a putative GDP-locked derivative. Then, the work explores how Rabl2 ensures the export of Hh proteins from cilia using an impressive combination of mass spec of isolated cilia, life imaging, density gradient centrifugation, generation of a Q80L knock-in mouse, IPs etc.. The authors conclude that GTP hydrolysis of Rabl2 licenses the BBSome and its associated cargoes to pass through the transition zone, a putative gate at the ciliary base, and exit the cilium.

This is a remarkable study, which re-defines and expands our understanding of Rabl2's role in ciliary protein transport. The usage of mouse models shows that Rabl2 is not required for general IFT, as previously reported, and that it is needed for Hh signaling and the export of Hh proteins from cilia. It also demonstrates that tagged "wild-type" Rabl2 undergoes IFT. The effects of the Q80L and other mutations are meticulously characterized. As I will outline below, I have some remaining doubts on the validity of the molecular model that proposes that the GTP cycle of Rabl2 regulates BBSome transit through the transition zone. I think this could be addressed by some rewriting and moderation of the conclusions.

Response:

We thank our reviewer for recognizing the strength and implications of the study. We have modified the main text, especially the discussion section, for better clarity. We have also toned down the wording in Discussion to reflect that we are proposing a model instead of stating a definite mechanism.

Main point

1. The key statement of the article is "(Rabl2's) GTP hydrolysis enables the outward TZ passage of cargo-loaded BBSomes with retrograde IFT machinery, whereas its persistent association leads to their shedding from IFT-B during the passing process and consequently ciliary retention." This would provide a mechanism for the recently proposed that the concept that the transit of (loaded) BBSomes through the TZ is a catalyzed event (Ye et al. 2018). Ye et al. observed only three such exit events over a 21 hour period. Here, the authors propose a critical role of Rabl2 GTP-cycle in catalyzing the exit. However, the actual exit events or the shedding of BBSomes from IFT were not observed directly in this study. Thus, I

think the statement above is not definitive and other explanations are possible.

Response:

We agree with our reviewer that what we propose is still a model, not a definite mechanism. As described below, we have provided additional results (Fig 5G-I and EV3) in favor of the model. We have toned down the wording in Discussion to avoid excluding other possibilities.

2. The supporting evidence is largely based on overexpression of the GTP-locked Q80L construct, which stably binds to IFT, causes an accumulation of BBS proteins in cilia etc. In such cells, the release from tagged BBS3 from cilia is decreased but IFT of IFT27GFP and BBS5-3xNG still occurs suggesting that it is specifically the exit step at the base of cilia, which is impaired. However, only the velocity of IFT was determined and it remains unclear whether the frequency of IFT and BBS transport are affected or not.

Response:

We appreciate the critical comments of our reviewer. In the revised manuscript, we have included quantification results on detailed behaviors of ciliary BBSome and IFT-B (Fig 5H, I; Videos EV2-EV5). We observed that the mean IFT frequency of 3NG-Bbs5 or Ift27-GFP puncta appeared to be slightly decreased in cilia of the Rabl2^{Q80L}-expressing cells (Fig 5H,I) but only the reduction in the retrograde frequency of 3NG-Bbs5 puncta (10.8 ± 5.0 counts/min vs. 15.1 ± 3.3 counts/min in the Rabl2-expressing cells) was statistically significant (Fig 5H). Comparing to RFP-Rabl2, RFP-Rabl2^{Q80L} did not significantly alter IFT velocities of BBSomes or IFT-B (Fig 5H, I). Retrograde 3NG-Bbs5 puncta were observed to reach the base of Rabl2^{Q80L}-positive cilia (Fig 5H and Video EV3), though the highly accumulated ciliary 3NG-Bbs5 did not allow convincing quantification on processivity of motile 3NG-Bbs5 puncta. When processivity of ciliary Ift27-GFP puncta was analyzed, we did not observe significant changes between the Q80L and the Rabl2 groups (Fig 5I). These results suggest that GTP-locked Rabl2 does not significantly alter ciliary IFT frequency, velocity, and processivity. Furthermore, the BBSome-containing retrograde IFT machineries can be assembled in the presence of Rabl2^{Q80L} and actively reach the ciliary base.

For quantifications on 3NG-Bbs5 behaviors (Fig 5H), we performed live imaging for both Q80L and Rabl2 groups during the revision period. Therefore, new images (Fig 5H) and videos (Video EV 2-3) are used to accompany the new quantification results in the revised manuscript. By contrast, the live imaging data for Ift27-GFP in both cell groups were acquired before in the same batch of experiments but we only presented results for the Q80L group in the initial manuscript. We only upgrade Fig 5I with new quantification results and images of a representative Rabl2/Ift27 double-positive cilium. We have also provided the time-lapse movie for the cilium in Video EV4 in the revised manuscript.

Also, the measurements of FLIP bleaching experiments are spread-out widely and I couldn't flow how the data were normalized and corrected for the loss of fluorescence especially considering that BBS proteins are enriched 8-12x in Q80L cilia. If BBS5-3xNG is also significantly enriched in cilia, a longer half-life of the signal does not necessarily mean that less BBS5 particles exited the cilium. Actually, the opposite might be true.

Response:

In the FLIP assays, the cytoplasmic pool of fluorescence was bleached so that 3NG-Bbs5 that entered the cilium after the bleach did not contribute to the ciliary fluorescence. In this way, the reduction of the ciliary fluorescence over time was attributed to ciliary retrieval of the protein. Theoretically the half-life (t1/2) is largely independent of concentration for certain molecules that diffuse homogenously into environment because higher concentrations will lead to higher diffusion rates. This is why the increased t1/2 is attributed to reduced diffusion through TZ in FLIP assays (Liew et al., 2014). In the revised manuscript, we have cited additional publications to aid understanding of the method.

3. Also, the GTP-locked Q80L form could have non-physiological negative effects, such as sequestering of GEFs, which has been described for other GTP-locked GTPases. The key question remains whether it is the physiological role of Rabl2 to permit BBSome exit from cilia. Most features of BBS mutants (polydactyly, kidney anomalies etc.) are also observed in hypomorphic IFT mutants. So, it is possible that loss of Rabl2 interferes with IFT and only indirectly affects BBSome traffic because the latter requires well-tune IFT.

Response:

We appreciate the comments of our reviewer. We believe that the D73GQ80L mutant is able to exclude the possibility of GEFs sequestration. It is also a GTP-locked mutant and able to enter cilia as efficiently as Rabl2^{Q80L}. Nevertheless, it does not cause ciliary accumulation of BBsome and GPCRs (Fig 6). Furthermore, results of Kanie and colleagues (Kanie et al., 2017) suggest that RABL2 is likely a GEF-independent GTPase. In this context, Rabl2 and its GTP-locked mutants might not even bind GEFs.

In the revised manuscript, we have performed further analyses to strengthen the idea that murine Rabl2 is not an important IFT regulator. In addition to the results mentioned in the response to the main point #2, we also quantified the ciliary Ift81 levels and did not observe significant difference between the *Rabl2*-deficient and wild-type MEFs (Fig 1L).

4. Indeed the phenotype of the Q80L mutation and knock-out are strikingly similar to Ift27-/-, as noted by the authors. So, I wonder if Rabl2 Q80L actually interacts with IFT25/27 blocking their ability to export BBSomes from cilia. To my knowledge, it is unclear if BBSome traffic by IFT is indeed impaired in the ift27 mutants. So it is unclear to me how loss of IFT27 is distinct from Rabl2Q80L. Instead of generally impairing IFT transport of BBSomes such mutations could prevent cargo-bound active BBSomes from binding to its IFT docking site.

Response:

We thank our reviewer for the insightful comments. We also suspected a direct Rabl2-Ift27 interaction when we observed the phenotypes of the Q80L KI/KI mice. We performed immunoprecipitation assays by co-expressing Rabl2^{Q80L} with GTP-locked or GDP-locked Ift27 mutant but failed to detect a convincing association, though either form of Ift27 strongly enriched Ift25 in the immunoprecipitates. Therefore, our results are in agreement with previous studies reporting that RABL2 interacts with the IFT74-IFT81, but not IFT25-IFT27, heterodimer (Kanie et al., 2017; Nishijima et al., 2017). Furthermore, as described in our response to the main point #2, Rabl2^{Q80L} did not strongly affect the ciliary

IFT of BBSomes (Fig 5H). Therefore, Rabl2^{Q80L} unlikely functions by preventing BBSome from binding to IFT-B. We thus tend to attribute the similarity between the Q80L KI/KI mice and the *Ift27*-deficient mice to the common inhibitory effects on ciliary retrieval of BBSome and its cargos.

5. Finally, the hedgehog defects of Bbs null and Rabl2 mutant animals are comparatively mild when compared to, e.g., some of the IFT-A and -B mutants. Thus, it is unclear whether the strong defects in the distribution of hedgehog proteins observed in ift27, Rabl2 and the Q80L mutant cells are similarly occurring in the corresponding animal models. All in all it seems that loss of Rabl2 and the Q80L allele are simply causing a minor defect in retrograde IFT with the known consequences on BBSome traffic and Hh signaling.

Response:

Comparing to the IFT machinery, BBSome-mediated trafficking indeed mainly functions as a regulatory mechanism for ciliary signaling. This is the major reason why interruption of BBSome-related functions usually results in genetic diseases after birth, whereas interruption of major functions of IFT machinery causes early embryonic lethality.

The moderate phenotypes of the *Rabl2*-deficient mice (Fig EV1) (Kanie et al., 2017) suggest that Hh signaling is not strikingly impaired. We observed increased Gli3-R levels in E11.5 limb buds and whole E10.5 embryos (Fig 2D, E). *Gli1* expression was still clearly visible in *Rabl2*-deficient embryos through X-gal staining (please refer to our response to Comment #7 of our reviewer #1). Comparing to in-vitro cultured cells, in-vivo situations are indeed much more complicated due to factors such as cell types, ligand concentrations and distributions, receptor/regulator levels, and cross-talking among signaling pathways.

All this said, this is an excellent work firmly establishing a connection between Rabl2 and Hh signaling, the BBSome and retrograde IFT. The authors could address this concern in the discussion section developing their conclusion or hypothesis step-by-step and provide the pro and contra arguments, as necessary.

Response:

We thank our reviewer for recognizing the strength of our study. We have extensively modified the discussion section in the revised manuscript according to the comments of our reviewers.

Minor points

1. Repeatedly, it is stated that the exit process applies to "loaded BBSomes". Is that meant to exclude a role of Rabl2 in the exit of unloaded BBSomes?

Response:

We have changed “loaded BBSome” to “the BBSome and its cargos” or the equivalent in the revised manuscript.

"To understand how Rabl2-GTP blocks the BBSome passage through TZ, we screened for a

Rabl2Q80L-based mutant that entered cilia without causing BBSome accumulation and identified the Rabl2D73GQ80L double mutant."
As mentioned in the discussion, a Rabl2-D73G mutant was previously described by Lo et al.. If this work inspired generation of this specific double mutant, it should be cited in the results as well.

Response:

In the revised manuscript, we have cited the related literature as requested.

p16, bottom: exit should be exist?

Response:

We are sorry for the typo. We have corrected it.

Fig. 5G) Since no 3D-SIM of control cilia is provided, I am not sure about the point of this figure.

Response:

We have provided 3D-SIM images of control (GFP-Rabl2) cilia in the revised manuscript. For better clarity and readability, we have moved the entire panel of images to Fig EV3B-C and left in Fig 5G only one set of 3D-SIM images for a typical Rabl2^{Q80L}-positive cilium to show the accumulation of SMO above TZ using another TZ marker, CEP290. To clearly demonstrate that ciliary BBSomes and GPCRs were accumulated above TZ, we have also clarified the spatial relationship among GFP-Rabl2 (distal appendages), acetylated tubulin (axoneme), CEP290 and CEP162 (markers located to the bottom of TZ) and have included a diagram to illustrate their ciliary localizations (Fig EV3A). These results clearly demonstrate that, while Rabl2^{Q80L} and IFT81 distributed both at distal appendages and in the ciliary shaft, BBS7, LZTFL1, SMO, and GPR161 distributed exclusively above TZ in Rabl2^{Q80L}-positive cilia.

Fig. 5H) in the second graph, the legend for the X-axis has moved.

Response:

We are sorry for the mistake that occurred during the submission process.

Referee #3:

This manuscript by Dr. Xueliang Zhu's lab reports the essential function of a small GTPase Rabl2 for fine-tuning cilia-dependent shh signaling, which is critical normal embryonic development and organismic homeostasis. By rigorous and careful studies of GTP-locked and GDP-locked mouse Rabl2 mutants, the authors reveal that the GTPase activity of Rabl2 controls the proper retrieval of ciliary GPCRs involved in shh signaling. Although there are some concerns remaining in this study as listed below, overall teh major conclusion of this manuscript is very interesting. the manuscript itself is well-written, with robust and clear data. My major comments are listed below:

1. Current studies suggest a two-diffusion-barriers model for the ciliary exit of signaling receptors, the transition fiber (TF) passage and transition zone (TZ) passage. A detailed single molecule imaging study revealed that more than 99% of activated GPCR that have crossed the TZ by BBSome train will bounce back to the ciliary compartment, likely dammed by the periciliary barrier (TF) (Fan Ye et al., 2018). RABL2 has been shown to colocalize with or very proximate (Inner PCM) to transition fibers. Knockdown of TF components compromised the ciliary base localization of RABL2 (Dateyama et al, 2019; Kanie et al.,2017), indicative of the association or dependence of RABL2 with TFs. The 3D-SIM imaging data (Figure 5G) also shows that GFP-Q80L actually can pass through the TZ and probably reach to TFs. Therefore, whether Rabl2 regulates the ciliary exit of GPCRs by controlling the BBsome mediated TZ passage or TF passage need to be further determined by super-resolution imaging. Specfically, Does GPCRs (e.g. Gpr161) can reach to the intermediate compartment between the TZ and TFs in Rabl2-Q80L-expressing cells?

Response:

We thank our reviewer for the insightful question. GPR161 was also accumulated above TZ in Rabl2^{Q80L}-expressing cells. In the revised manuscript, we have provided 3D-SIM images of GPR161 as requested. For better clarity and readability, we have moved the entire panel of images to Fig EV3C and left in Fig 5G only one set of 3D-SIM images for a typical Rabl2^{Q80L}-positive cilium to show the accumulation of SMO above TZ using another TZ marker, CEP290. Following the request of our Reviewer #2, we have also presented 3D-SIM images of control (GFP-Rabl2) cilia in Fig EV3B. Furthermore, to clearly demonstrate that ciliary BBSomes and GPCRs are accumulated above TZ, we have also clarified the spatial relationship among GFP-Rabl2 (distal appendages), acetylated tubulin (axoneme), and CEP290 and CEP162 (markers for the bottom of TZ) and have included a diagram to illustrate their relative ciliary localizations (Fig EV3A).

These results clearly demonstrate that, while Rabl2^{Q80L} and IFT81 distributed both at distal appendages and in the ciliary shaft, BBS7, LZTFL1, SMO, and GPR161 distributed exclusively above TZ in Rabl2^{Q80L}-positive cilia, strengthening the idea that they were blocked by TZ but not by the periciliary barrier.

2. The authors show that Rabl2-Q80L does not affect ciliary abundance of IFT components. However, this does not necessarily mean that IFT function is not affected. The velocity and processivity of IFT machinery in Rabl2-Q80L and proper control cells should be examined.

Response:

We actually imaged ciliary Ift27-GFP puncta in living cells expressing respectively RFP-Rabl2 and RFP-Rabl2^{Q80L} but presented only the data for the Rabl2^{Q80L} group in our initially submitted manuscript. Following the request, we have included the Rabl2 group as control in the revised manuscript. As shown in Fig 5I, we did not observe significant differences between the two groups in velocity, frequency, and processivity of Ift27-GFP puncta. These results suggest that the GTP-locked mutant does not significantly alter IFT.

3. The conclusion that "Rabl2-GTP enters cilia through IFT" is overstated (Figure 4). Indeed, the GTP-bound Rabl2, but not GDP-bound form, has strong affinity to IFT-B complex. Direct

evidence needed to show that disrupted IFT would affect the cilia entry of Rabl2. The authors should also consider how to reconcile with the previous study that GTP-RABL2B triggers cilia import of IFT-B (Kanie et al., 2017). Knocking down may IFT-B components produce truncated cilia, which could be used for the experiment.

Response:

We are sorry for having not clearly presented this part of the results. The conclusion is based on both the previous reports (Kanie et al., 2017; Nishijima et al., 2017) and our results. RABL2-GTP has been shown to be specifically recruited to distal appendages by CEP19, where it binds to IFT-B (Kanie et al, 2017; Nishijima et al., 2017). The consistent localization of Rabl2 and Rabl2^{Q80L} at distal appendages (Fig EV2 and EV3) suggests that Rabl2-GTP is recruited to distal appendages by the same mechanism to interact with IFT-B. The only difference lies in the scenario after this: Kanie and colleagues propose that only IFT-B enters cilia, but we speculated that Rabl2-GTP entered cilia together with IFT-B. To clarify this discrepancy, we confirmed that wild-type Rabl2 was indeed able to enter cilia (Fig 4A-E). Importantly, 3NG-Rabl2 puncta displayed rapid anterograde and retrograde movements along cilia, a feature of the bidirectional intraflagellar transport (Fig 4C and Video EV1). Then we confirmed that only the GTP-binding form of Rabl2 interacts with IFT-B (Fig 4F-I), similar to the case of RABL2 (Kanie et al, 2017; Nishijima et al., 2017). Therefore, we revise the model of Kanie and colleagues and propose that Rabl2-GTP enters cilia through IFT and functions in the ciliary shaft to regulate the proper turnover of ciliary GPCRs. Such a model is also supported by the finding that the interaction of RABL2-GTP with CEP19 and the IFT74-IFT81 heterodimer is mutually exclusive (Nishijima et al., 2017). In the revised manuscript, we have modified the texts in this part and discussion accordingly to improve the clarity of the presentation.

We feel that these lines of evidence are strong enough to support the statement of an IFT-dependent entry of Rabl2-GTP. In comparison, knocking down IFT-B components would unlikely provide a better support: complete depletion of core IFT-B components would abolish cilia growth and preclude the suggested analysis, whereas results achieved through incomplete depletion would hardly be convincing.

Although human RABL2 appears to play an important role in ciliary import of IFT-B (and thus ciliogenesis), our results argue that murine Rabl2 is largely dispensable for the IFT-B import. The viability of the *Rabl2*-deficient mice (Fig EV1) and the normal ciliogenesis of the *Rabl2*-deficient tissues and cells (Fig 1E-J) are strong solid evidence. In the revised manuscript, we quantified ciliary Ift81 levels and did not observe significant difference between the *Rabl2*-deficient and the wild-type MEFs (Fig 1K, L). Furthermore, as described in our response to the major comment #2, our results suggest that the GTP-locked mutant does not significantly alter IFT (Fig 5I). Therefore, we propose that Rabl2 mainly functions in cilia to modulate ciliary signaling and such a role is also conserved in RABL2 because RABL2^{Q80L} also enters cilia and induces ciliary accumulations of BBS4 (Kanie et al., 2017) and GPCRs (GPR161 and HTR6) (Dateyama et al., 2019). Following the request of our reviewer, we have extensively modified the discussion section to reconcile our findings on Rabl2 with previously reports on its human orthologues.

4. Given the functional redundancy of human RABL2A and RABL2B and its evolutional conservation, it is quite surprising that murine Rabl2 is dispensable for ciliogenesis. Is there any possibility that a redundant player for Rabl2 exists in mouse cells? Although the net ciliation ratio was not affected, to comprehensively understand the functional role of Rabl2 in ciliogenesis, cilia stability and cilia length in Rabl2-/- cells need to be carefully examined.

Response:

Indeed, human *RABL2* has two paralogues but murine *Rabl2* is a single gene. Why human RABL2 displays a more important function in IFT-B entry is intriguing. Although we demonstrate that Rabl2 is largely dispensable for IFT-B entry, our results do not exclude the possibility of a subtle role. RABL2 might become more important in this through evolution. Alternatively, as the *Chlamydomonas* orthologue is critical for flagella formation (Nishijima et al., 2017), evolution might somehow relieve murine Rabl2 of this role. In addition, it is also worth noting that current studies on RABL2 are solely based on RPE1 cells. Results in other human cells, especially primary cells, still await future investigations. These discussions have been accordingly included into the revised manuscript.

Following the request of our reviewer, we measured cilia lengths of $Rabl2^{+/+}$ and $Rabl2^{-/-}$ MEFs and observed a slight reduction in the $Rabl2^{-/-}$ MEFs (2.3 ± 0.6 μm vs. 2.5 ± 0.7 μm in wild-type MEFs; Fig 1J). In response to comments of our reviewers #1 and #2, we also quantified ciliary intensity of Ift81, a key subunit of IFT-B, and did not observe significant difference between the ko and wt groups (Fig 1K, L). As to cilia stability, our reviewer might already notice that we examined multiple tissues of 8-week-old ko and wt mice but did not observe obvious differences in ciliary number and length (Fig 1F-H).

5. As Rabl2Q80LD73G markedly reduces its association with IFT-B, and IFT-B is essential for ciliary entry of Rabl2 as claimed by the authors, it is quite surprising that the Rabl2Q80LD73G mutant still enters the cilium and show strong anterograde and retrograde IFT (Figure 6). What is the explanation?

Response:

We thank our reviewer for this question. As explained in our response to the major comment #3, results of other groups and ours suggest that Rabl2-GTP interacts with IFT-B at distal appendages and enters cilia together with IFT-B. As $Rabl2^{D73GQ80L}$ still interacted with IFT-B, its presence in cilia is expected to follow the same mechanism, which is further confirmed by its IFT behaviors (Fig. 6B; Video EV6). In ciliary shaft, however, $Rabl2^{D73GQ80L}$ is expected to display dynamic interactions with IFT-B, undergoing repetitive dissociation and association. As each IFT train contains multiple IFT-B complexes, multiple Rabl2 molecules are expected to reside in each motile punctum and display the dynamic behaviors independently of each other. Therefore, their steady-state total fluorescence intensity may not look strikingly changed during live imaging. Nonetheless, this is only a plausible explanation. Future investigations are certainly required to understand detailed mechanisms.

1st Revision - Editorial Decision 1st Oct 2020

Thank you for submitting a revised version of your manuscript. Your study has now been seen by all original referees, who find that most of their main concerns have been addressed and are now broadly in favour of publication of the manuscript. There now remain only a few mainly editorial issues that have to be addressed before I can extend formal acceptance of the manuscript:

1. Please address the remaining minor points from reviewers #1 and #2.

Referee #1:

The authors have satisfactorily responded to most of my previous comments.

I do have a few comments on Gli3-FL/R quantification in Figures 2 and 3 (previous comment #7). As shown now in the updated Figures, the authors seem to be quantifying relative levels of either FL or R forms by normalizing the wild type (unstimulated) bands to 1. The ratio however should be calculated from the raw FL to R levels of that particular sample, and not considered 1, as the authors have currently done. The authors should revise the values of the ratios accordingly. They can also show the relative FL and R values separately with respect to wild type (normalized to 1) separately.

The authors mention in the legends to Fig 2D, E that Gli3R production is reduced in E11.5 hind limb buds and E10.5 mouse embryos. I think the authors are meaning Gli3R/Gli3FL ratios in their statement. The Gli3R levels are not changing much between respective samples.

The Gli1 levels in ko limbs (Reviewer Fig 3) do not show a gross decrease (as seen in the MEFs). Rather, the preaxial polydactyly in hindlimbs probably arises from high Hh signaling in the limb buds. Reduction in GliR with respect to GliA is often reflected by anterior expansion of posteriorly expressed HoxD genes in the limb buds, although, these experiments are well beyond the scope of the current paper (for e.g. please see PMID 15930098 and 32540122).

Referee #2:

Rabl2 GTP hydrolysis licenses BBSome-mediated export to fine-tune ciliary signaling by Duan et al.

All my concerns have been addressed in a satisfactory manner. The addition of new data made an excellent manuscript even stronger.

Typos:
also enters (the) primary cilium OR also enters primary cilia

and membranes receptor accumulations

Referee #3:

I have reviewed the revised manuscript and am convinced this is a high-quality study, with all my concerns properly addressed by the authors. The conclusion is fully justified by evidences presented.

Responses to reviewers' concerns

Referee #1:

The authors have satisfactorily responded to most of my previous comments.

Response:

We thank the reviewer for appreciating our effects.

I do have a few comments on Gli3-FL/R quantification in Figures 2 and 3 (previous comment #7). As shown now in the updated Figures, the authors seem to be quantifying relative levels of either FL or R forms by normalizing the wild type (unstimulated) bands to 1. The ratio however should be calculated from the raw FL to R levels of that particular sample, and not considered 1, as the authors have currently done. The authors should revise the values of the ratios accordingly. They can also show the relative FL and R values separately with respect to wild type (normalized to 1) separately.

Response:

In the revised manuscript, we have presented the relative Gli3 band intensities and the FL/R ratios in Fig 2D, 2E, and 3H following the request of our reviewer. In Fig 2E and 3H, we have presented the FL/R ratios from different sets of independent experiments separately to better show changes that are consistent among the experiments. We have modified the text accordingly.

The authors mention in the legends to Fig 2D, E that Gli3R production is reduced in E11.5 hind limb buds and E10.5 mouse embryos. I think the authors are meaning Gli3R/Gli3FL ratios in their statement. The Gli3R levels are not changing much between respective samples.

Response:

We have modified the legends as requested.

The Gli1 levels in ko limbs (Reviewer Fig 3) do not show a gross decrease (as seen in the MEFs). Rather, the preaxial polydactyly in hindlimbs probably arises from high Hh signaling in the limb buds. Reduction in GliR with respect to GliA is often reflected by anterior expansion of posteriorly expressed HoxD genes in the limb buds, although, these experiments are well beyond the scope of the current paper (for e.g. please see PMID 15930098 and 32540122).

Response:

We appreciate the comments and suggestions that may shed light on our future researches.

Referee #2:

Rabl2 GTP hydrolysis licenses BBSome-mediated export to fine-tune ciliary signaling by Duan et al.

All my concerns have been addressed in a satisfactory manner. The addition of new data made an excellent manuscript even stronger.

Response:

We thank the reviewer for appreciating our effects.

Typos:
also enters (the) primary cilium OR also enters primary cilia
and membranes receptor accumulations

Response:

We carefully proofread the manuscript and have corrected typos to the best our knowledge.

Referee #3:

I have reviewed the revised manuscript and am convinced this is a high-quality study, with all my concerns properly addressed by the authors. The conclusion is fully justified by evidences presented.

Response:

We thank the reviewer for appreciating our effects.

2nd Revision - Editorial Decision 23rd Oct 2020

Thank you for addressing the remaining minor issues. I am now pleased to inform you that your manuscript has been accepted for publication.

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: Xueliang Zhu
Journal Submitted to: EMBO journal
Manuscript Number: EMBOJ-2020-105499

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:

- 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 $\chi 2$ 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?	No sample-size calculation was performed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The sample size was included for animal studies.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	For FLIP assay, to be more unbiased, we excluded the cilia with the initiate fluorescence intensity of 3NG-Bbs5 larger than 50,000 A.U..
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.	Cells and animals were randomly allocated only based on the treatment or genotyping.
For animal studies, include a statement about randomization even if no randomization was used.	The statement about randomization was included.
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.	Cells and animals were randomly allocated only based on the treatment or genotyping.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The statement was included in the paper.
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.	The unpaired t-test was used to access differences with no assumption of our data.
Is there an estimate of variation within each group of data?	Yes. The estimate of variation was shown with standard deviation (SD).

USEFUL LINKS FOR COMPLETING THIS FORM

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

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-tumo

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 have been accurately annotated.
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, IMCD3 and HEK293T were from ATCC and were routinely tested for mycoplasma contamination.

* 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.	4-week old and P0 mice were used for primary cell culture (mTECs and mEPCs, respectively). The detail housing, husbandry and source of mice were provided in the "materials and Methods".
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The statement was included in the "Materials and Methods".
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.	All the relevant aspects of animal studies were adequately reported.

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	"Data Availability" section was provided at the end of the Materials & Methods.
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.