JCB

LOSS OF ARL13 IMPEDES BBSOME-DEPENDENT CARGO EXPORT FROM CHLAMYDOMONAS CILIA

Jin Dai, Gui Zhang, Rama Alkhofash, Betlehem Mekonnen, Sahana Saravanan, Bin Xue, Zhen-Chuan Fan, Peiwei Liu, and Karl Lechtreck

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February 17, 2022

Re: JCB manuscript #202201050

Dr. Karl Lechtreck
University of Georgia
Cellular Biology 120 Cedar Ave, 635 Biological Science Bldg
Athens, GA 30602

Dear Dr. Lechtreck,

Thank you for submitting your manuscript entitled "CHLAMYDOMONAS ARL13 FACILITATES BBSOME-CARGO INTERACTION FOR PROTEIN EXPORT FROM CILIA." Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

You will see that the reviewers find the premise of your work interesting and appropriate for JCB but also raise significant concerns with some aspects of the study and ask for additional evidence to support the major conclusions. Their detailed comments suggest experiments and revisions that would render the study more suitable. We feel that all comments are reasonable and would need to be fully addressed in revision.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. If you intend to submit a revision we ask that you first send us a detailed revision plan with a point-by-point response explaining how you will address each comment. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that implementation of measures that limit spread of COVID-19 also pose challenges to scientific researchers. Therefore, JCB has waived the revision time limit. If you will require more time we recommend that you reach out to the editors to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

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Text limits: Character count is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

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IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

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Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually

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Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Maxence Nachury, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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

In this study, the authors investigated the role of Chlamydomonas ARL13 by conducting various experiments. Based on the results of these experiments, the authors concluded that Chlamydomonas ARL13 participates in the export of signaling proteins (at least PLD) from cilia via the IFT/BBSome pathway by enabling BBSome-cargo interactions. The individual biochemical and TIRF experiments are well done, and I think the results obtained are generally valid. However, it is difficult to connect these results to the authors' argument, as there is a lot of speculation involved in drawing this conclusion. I also think that the comparison with ciliary protein trafficking in mammals is insufficient. Therefore, I do not think that the current version is of a high enough quality to be published in JCB because of several ambiguities as described below. However, if these problems can be solved, I think it is possible to publish this paper in JCB.

Major points.
1. Some lines of evidence are consistent with the authors' claim (for example, phenotypic similarity between arl13 and bbs mutants and their hierarchy; & arl3 mutant). However, there is no evidence for direct interaction of ARL13 with IFT or BBSome. I think there is too much speculation to connect ARL13 to cargo loading to IFT/BBS, based only on indirect evidence.
2. Based on the results of experiments with ARL13F54A, the authors associated the function of ARL13 to its ARL3-GEF activity as described 'ARL13 might regulate its own binding to IFT via its GEF activity'. However, there were no experiments or no discussion of how ARL3 is involved in the regulation of interaction of ARL13 or cargo with the IFT/BBSome system.
3. In the mammalian system, ARL3 is involved in release of Rho-GDI-like factors, Unc119 and PDE6delta, from lipidated membrane proteins, and ARL13b is involved in localization of INPP5E (phosphoinositide phosphatase) to the ciliary membrane as well as serves as an ARL3-GEF. Despite the fact that this study dealt with lipidated membrane proteins such as PLD and FAP12 (and those listed in Table 1), there were no experiments or no discussion from this viewpoint. There was no mention of whether there is a Rho-GDI-like system in Chlamydomonas or not.

Minor points
1. There are two Table S1 (page 26 and page 39). Probably, that on page 39 is Table 1.
2. Typographical errors:
Page 8, line 14: Fig. S2A (NOT Fig. 2SA)
Page 9, line 5 from the bottom: Delete 'on'.
Page 10, line 4 & page 39: arl13 (NOT arl13b)

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

In this manuscript Dai et al. use Chlamydomonas as a model organism to study the function of ARL13 in ciliary membrane protein composition and transport. The vertebrate ARL13 homolog, ARL13B, has been well studied by several groups and

mutations in ARL13B has been linked to defective Hedgehog signaling and ciliopathies in human and mouse.
Here the authors characterize a Chlamydomonas null mutant in ARL13 (arl13) and show using biochemical approaches (cilia isolation/fractionation, mass spectrometry, western blotting) that ARL13 loss leads to abnormal ciliary accumulation of some membrane proteins (e.g. PLD), and reduced ciliary levels of others (e.g. FAP12). They also show that endogenous and tagged ARL13 is localized in the ciliary membrane fraction, and occasionally/rarely moves by IFT, as observed before. In previous work the authors nicely showed that PLD is a cargo for the BBSome during ciliary protein export, hence they decided to focus here on the possible connection between ARL13, PLD and the BBSome. By using various Chlamydomonas mutant and rescue lines, combined with biochemical analysis, live cell/TIRF imaging and phenotype analysis (phototaxis assays), the authors provide evidence to suggest that ARL13 is required for mediating PLD association with the BBSome for its ciliary export, in turn affecting phototaxis. Loss of ARL13 seems not to grossly affect IFT/BBSome transport itself.

While the topic and results should be of great interest to cell- and developmental biologists, and the paper in principle would be suitable for JCB, I have several important concerns that need to be addressed by the authors before this manuscript is suitable for publication. Particularly, I find that lack of quantitative analysis of key western blot data (and some other data as well) is problematic, as it means that some of the main conclusions drawn by the authors are not fully justified by the data. Furthermore, some of the phenotype- and (partial) rescue data provided are confusing and require clarification. My specific comments are listed below.

Main comments:

1) Issues with western blot data and interpretation thereof:

A substantial fraction of the figures includes western blot data comparing ciliary levels of various relevant proteins, in wild type and different mutant and rescue lines (e.g. Figures 1B, 1F, 2A, 3A, 3B4B, 4D5A, 5B, S1C, S1D, S2A-E, S3A, S3B, S5B). Based on visual inspection of these blots, the authors draw several (major) conclusions regarding relative ciliary levels of different proteins in the various strains. However, for some of the blots mentioned, the interpretation of the relative band intensities is not as obvious as postulated by the authors, especially because the loading is sometimes uneven, and no quantification of band intensities is provided.

For example, in Figure S2B it looks like GT335 might be decreased in arl13 mutant cilia (considering the elevated IC2 signal), which is in direct contrast to what the authors write on bottom of page 8, but which would be in line with that reported by others in e.g. C. elegans.

Another example is Figure S3A where it is hard to appreciate the increase in PLD levels in arl13 from 40-120 min (given reduced IC2 signal at 40 min), whereas Arl13 levels in the bbs4-1 mutant looks reduced initially (40 min) and then gradually increases. The latter result suggests that the BBSome might be needed to keep ciliary levels of ARL13 low, which agrees with the blot in Fig. S3D. On the other hand, the increased ciliary ARL13 levels seen in the bbs4-1 mutant is apparently not rescued by BBS4-GFP (Figure 3B). The authors comment on this on page 15, but the results are quite confusing and moreover, blots from the different strains shown in Figure S3A look to be from different gels (?) making direct comparison of band intensities nearly impossible for the reader. In Figure 3B it is hard to compare endogenous BBS4 levels with BBS4-GFP as different antibodies were used to probe for the two. In this blot IC2 band intensity also looks lower in WT than in other lanes, again making comparison difficult.

Finally, in Figure S1D it looks like there is ALR13 in the last lane, but authors claim on page 6 bottom there is not. In Figure S1D, the labeling above the lanes is also misaligned.

There are similar issues with some (but not all) of the other western blot figures. In summary, the authors need to carefully quantify the most central western blot results and make sure their conclusions are justified by the data provided.

2) Figure 3D: these results are a bit confusing, is the apparent difference between the two strains statistically significant? Could the (apparent) reduced IFT frequency in the arl13 mutant be related to possibly altered tubulin polyglutamylation (see comment above to Figure S2B)? Related to this: page 10 bottom and page 11 lines 7-8, are the authors 100% sure IFT/BBSome traffic is normal in the arl13 mutant strain? It is not so obvious based on the data shown in Figure 3D.

3) Figure 4C and page 13, 5 lines from top: the phototaxis data are not easy for a non-expert to interpret, and without quantitative analysis, how can authors claim that the arl3 mutant phototaxed "less efficiently" than control cells?

4) Rescue experiments: it is unfortunate that the phototaxis phenotype of the arl13 mutant cells cannot be fully rescued by expression of ARL13-mNG (Figure 1D and Figure S1H), which according to the authors could be due to low level expression of the transgene compared to endogenous ARL13 (Fig. 1B and 4D). Since the ARL13F53A-NG rescue construct is expressed at higher level than the wild type version (Figure 4C), did authors try to do phototaxis assay with this strain? Related to this and page 8 first paragraph, could the authors test if PLD export from cilia is delayed in the arl13 ARL13-mNG strain?

Minor comments:

Figure S1H: numbers are very small and hard to read.

Page 6, 2 lines from bottom: the authors cannot use the term "quantitatively" when the western blot results in Fig. S1D were not quantified.

Page 7 middle: where is the data for the MS analysis referred to here? Is it the same as the data shown in the Table S1 located on page 39? Please clarify.

Page 8 line 2: "inversion" should be "Inversin" as this compartment refers to the proximal region of the cilium occupied by the Inversin protein.

Page 9, 5 lines from bottom: "causes on accumulation" should be "causes accumulation".

In many places throughout the text, arl13 mutant cells or arl13 mutant cilia are referred to simply as "arl13". Please specify whether you mean arl13 mutant cells or cilia.

Page 16 top: the statement "In arl13 mutants, PLD failed to bind to the BBSomes" is not justified by the data provided, the authors only show accumulation of PLD in arl13 mutant cilia, not that PLD cannot bind to BBSomes. To justify this statement, authors would need to perform IP experiments or similar. Further down on the same page: "BBSome composition and traffic were apparently normal in arl13" is also not fully justified, given the data shown in Figure 4D (but see comment to this figure above).

Mislabeling of Tables:

Page 26-28 contain three tables that are labeled Table S1, S2 and S3, respectively. Page 39 also includes a table named Table S1 (i.e. there are two Table S1). Page 8 bottom refers to a "Table 1" but there is no such table included, and the same goes for "Table 3.2", "Table S3.3", "Table 3.3" mentioned on page 20 and "Table 3.4" mentioned on page 23.

Table S2: it would be more informative for the reader if this table had a bit more information about what the different primers were used for, rather than just the numbers (even though some explanation is provided in the methods section).

Reference list:

Mariani et a. 2016 and Yan and Shen 2021 are incomplete.

Reviewer #3 (Comments to the Authors (Required)):

In their manuscript, "CHLAMYDOMONAS ARL13 FACILITATES BBSOME-CARGO INTERACTION FOR PROTEIN EXPORT FROM CILIA " Dai et al. use a combination of genetic mutants, biochemical analysis and sophisticated live imaging to investigate the function of ARL13 in Chlamydamonas. In the context of previous work in other mutants, the authors conclude that "Chlamydomonas ARL13 enables BBSome-cargo interactions to ensure the export of signaling proteins from cilia." This is important work as it advances the field's understanding of what exactly ARL13 might do, which is currently not understood. Overall, the work is rigorous in its experimentation with appropriate controls and replicates. The significance of the work is underscored by the fact that it provides a plausible mechanism through which to explain some of the phenotypic overlap and distinctions among various ciliopathies, assuming that the function of ARL13 is conserved.

There are several points that must be addressed:
1) Chlamydamonas is the work horse organism of ciliary biologists and I cannot say enough about how much biology we understand due to its study. Indeed, there is so much conservation from Chlamy to human that underlies my excitement about this work. That said, the authors must make clear the distinction of ARL13 from ARL13A (about which we know almost nothing) and ARL13B (about which we know slightly more than nothing). I recognize that conflating ARL13 and ARL13B in Chlamydomonas was initiated in the Wittinghofer work from 2015 where they designated Chlamydamonas ARL13 as ARL13B in clear conflict with any nomenclature guidelines. This is not simply semantic as there may well be distinct evolution of ARL13 function to ARL13A and 13B. This work helps lay the groundwork for testing CrARL13 function in additional organisms. (Note, worm ARL13 is referred to incorrectly as ARL13B in at least one place I saw)
2) The Chlamy arl13 allele needs to be more clearly defined/explained. The authors treat it as a null but are rather vague about the exact data supporting that assertion. Where is the insertion relative to the G motifs? Are they all there? What data do the authors have to state in the legend of Figure 1, "We have no evidence that a residual truncated ARL13 is expressed."? It appears that the antigen for the antibody is at the C terminus of the protein so would not detect a N-terminal truncated protein. What's up?

3) Where does ARL13 localize in Chlamy? Does the ARL13-mNeon reflect endogenous protein localization? The authors have an antibody- where do they see it via IF? This addresses the broader issue of where ARL13 functions in Chlamy. The final conclusion in Chlamy is that ARL13 in the matrix helps attach membrane proteins onto the BBSome but is that where the authors see it? What about Chlamy in which the membrane is removed- any change? On the flip side, previous work from the Barr and Blaque labs showed ARL13B functions at the ciliary axoneme yet the authors detect no defects in the Cr arl13 mutant axonemes. These are examples of where the manuscript appears to use ARL13 and ARL13B similarities only when convenient. ARL13B is not detectable on mammalian cilia where the membrane is stripped off. The authors should discuss the possible reasons for such differences and how it might impact the model they propose for mammalian ciliary traffic.
4) The authors do make a point at the end to acknowledge the role of ARL13 in ciliary protein entry as well as the model they put forth. Yet they use much stronger language in the abstract and manuscript. Rather than propose that "ARL13 enables BBSome-cargo interactions to ensure the export of signaling proteins from cilia", they conclude it. I very much like the model but it should be presented as a model. I think it is also possible that ARL13 could control ciliary entry of a factor X that is directly or indirectly responsible for the BBSome-cargo interactions. Obviously, any of the 4 proteins identified in the mass spec to be depleted in arl13 mutants would be prime candidates. Do the authors have data to refute such a possibility?
5) Along these lines, it is unclear whether there are literally only 4 enriched and 4 depleted proteins in the arl13 mutants or if the authors are only reporting 8 proteins and others are unreported. The authors should clarify.
6) I don't think the authors are trying to say that the data supporting ARL13B as an ARL3 GEF is untrue on Page 13. Might be worth mentioning that the data would support there being additional ARL3 GEFs in vivo.
7) Related to point 1, proper nomenclature should be used throughout- human genes are all capital letters in italics, mouse genes only capitalize first letter etc. This will help clarify what the authors are referring to. Adding clear designations on the proteins could help. For example, on page 7, I am interpreting the ARL13 fused to mNeon to be Chlamy Arl13. (and there's a future experiment to use mammalian ARL13A and ARL13B to see whether both rescue or whether distinct functions evolved to each)
8) The references require detailed attention. Many are inaccurate. Some examples in regards to mammalian ARL13B include the Caspary 2007 reference describing work in mouse that did not connect the gene to Joubert syndrome and the Gigante 2020 manuscript that did not address the question of Arl13 in Chlamy much less show that there are 2 ARL13 proteins in metazoans. The correct references are needed. Additionally, there are papers on ARL13 in worm from the Barr lab showing that ARL13 plays critical roles in cilia structure and ciliary traffic- as well as the cited one from the Hu lab linking ARL13 to IFT. As the work presented here focus on ARL13, it is important the authors present the context accurately and discuss how the data might be reconciled (technical distinctions, species differences etc).

Re: JCB manuscript #202201050

Point-by-point response to the referees' comments

We thank the referees for their through work and many good suggestions, which helped us to improve the manuscript. The referees' critiques triggered several additional experiments, which allowed us to provide a better picture of ARL13's connections to the IFT/BBS pathway. The manuscript was rewritten accordingly.

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

In this study, the authors investigated the role of Chlamydomonas ARL13 by conducting various experiments. Based on the results of these experiments, the authors concluded that Chlamydomonas ARL13 participates in the export of signaling proteins (at least PLD) from cilia via the IFT/BBSome pathway by enabling BBSome-cargo interactions. The individual biochemical and TIRF experiments are well done, and I think the results obtained are generally valid. However, it is difficult to connect these results to the authors' argument, as there is a lot of speculation involved in drawing this conclusion. I also think that the comparison with ciliary protein trafficking in mammals is insufficient. Therefore, I do not think that the current version is of a high enough quality to be published in JCB because of several ambiguities as described below. However, if these problems can be solved, I think it is possible to publish this paper in JCB.

Major points.
1. Some lines of evidence are consistent with the authors' claim (for example, phenotypic similarity between arl13 and bbs mutants and their hierarchy; & arl3 mutant). However, there is no evidence for direct interaction of ARL13 with IFT or BBSome. I think there is too much speculation to connect ARL13 to cargo loading to IFT/BBS, based only on indirect evidence.

This point is similar to comments from referee 3. Our previous conclusion/model that ARL13 could be a loading factor for the BBSome, has been revised and is now discussed as just one of several possibilities. This model was based on our observation that ARL13-NG and especially ARL13(F53A)-NG travel (at least occasionally and transiently) on IFT trains, which we consider evidence for direct or indirect ARL13-IFT interaction. However, we now expressed ARL13-NG and ARL13(F53A)-NG in the *arl13 bbs4* mutant background and observed similar IFT frequencies of those proteins as in cells possessing intact BBSomes. Thus, IFT of ARL13-NG is not BBSome dependent and our data provide no evidence for ARL13-BBSome interactions. We rewrote the discussion to reflect the new data and to make it clear that it remains unknown how ARL13 promotes IFT of PLD.

To further explore the relation between ARL13, IFT and the BBSome, we included a new experiment using the conditional IFT motor mutant fla10-1. When IFT is switched off, (non-phopho) ARL13 accumulates in cilia (see figure 5).

2. Based on the results of experiments with ARL13F54A, the authors associated the function of ARL13 to its ARL3-GEF activity as described 'ARL13 might regulate its own binding to IFT via its GEF activity'. However, there were no experiments or no discussion of how ARL3 is involved in the regulation of interaction of ARL13 or cargo with the IFT/BBSome system.

A similar point was also raised by referee 3. Actually, a *Chlamydomonas arl3* mutant was analyzed. It has the same biochemical defects (i.e., reduction of FAP12 and accumulation of PLD) as the *arl13* mutant. This supports findings by others that ARL13 and ARL3 are a functional pair, which was added to the discussion: "*PLD accumulates in a Chlamydomonas arl3 mutant supporting the idea that ARL13 and ARL3 form a functional pair during the regulation of BBSome-dependent export of PLD as they apparently do in protein import*"). Our co-author Zhen-Chuan Fan informed me that his lab is working on *Chlamydomonas* ARL3 and has a forthcoming paper. Therefore, we think a full-scale investigation of ARL3 is outside the scope of this manuscript.

Additional imaging confirmed that IFT of ARL13(F53A)-NG while rare is more processive than that of ARL13-NG (Table S1, Fig. 5F). The reason for this is unknown and the idea that the loss of its GEF activity could make the transport more processive is now only briefly mentione din the Results section ("*While the reason for the more stable association of ARL13^{F53A}-NG with IFT is unknow, it is possible that ARL13's GEF activity regulates the duration of its transport.*"). We now acknowledge repeatedly that bona fide long-distance IFT of both ARL13-NG and ARL13(F53A)-NG was exceedingly rare raising doubts if these transport events are related to PLD loading onto the BBSome.

However, BBS3, which based on several lines of evidence, is a known interactor and loading factor of the BBSome, does not move by IFT in *Chlamydomonas* (Jin et al., 2010; Liu et al., 2021). Further, using in vitro assays, Klink et al. showed that ARL6/BBS3 does not impact BBSome-cargo interactions. Nevertheless, *Chlamydomonas* BBS3 is need for BBSome-dependent PLD/cargo export. Thus, the rare IFT of ARL13-NG observed could be the tip of an iceberg with many more ARL13-IFT contacts occurring but being to brief to give proper IFT traces. The small GTPase RABL2, a known IFT-B binding protein, apparent is also involved in ciliary cargo export via the BBSome showing that RABL2-IFT-B contacts can impact BBSome-dependent cargo export (Duan et al., 2021; Kanie et al., 2017; Nishijima et al., 2017). Thus, there remain many unknowns and inconsistencies even in the widely accepted models of how GTPases affect BBSome/IFT transport; the discussion was modified accordingly.

3. In the mammalian system, ARL3 is involved in release of Rho-GDI-like factors, Unc119 and PDE6delta, from lipidated membrane proteins, and ARL13b is involved in localization of INPP5E (phosphoinositide phosphatase) to the ciliary membrane as well as serves as an ARL3-

GEF. Despite the fact that this study dealt with lipidated membrane proteins such as PLD and FAP12 (and those listed in Table 1), there were no experiments or no discussion from this viewpoint. There was no mention of whether there is a Rho-GDI-like system in Chlamydomonas or not.

Here are the actual data: Human UNC119 has a likely *Chlamydomonas* ortholog based on reciprocal blast search (Blast score 9e-52 over 96% of the sequence). A *Chlamydomonas* UNC119 mutant is currently unavailable.

PDE6D has a single weak blast hit in the *Chlamydomonas* genome (score 6e-8). This hit is annotated as a PDE6delta and in reciprocal Blast hits UNC119a (score 9e-52) and not PDE6D.

INPP5E Blast finds a set of three large (>1,600 aa) proteins with putative inositol polyphosphate 5-phosphatase domains (Blast 6e-26 over the ~200 residue long catalytic domain). In reciprocal blast, the highest scoring of these three hits aligns best which with other INPP5 sub-types, based on the short GMP-PDE delta domain.

To address this point, we briefly discussed that UNC119 is present in *Chlamydomonas* and thar the observed reduction of FAP12 in *Chlamydomonas arl3* and *arl13* mutants suggests that a similar ARL13-ARL3-Unc119-based protein import pathway appears to be present in *Chlamydomonas* ("*In contrast, ARL13, together with ARL3 and carrier proteins such as UNC119 has been mostly associated with ciliary protein import (Gotthardt et al., 2015).* AND *These proteins could enter cilia using the previously described ARL13/ARL3/UNC119 import system, a participation of IFT in this shuttle remains unknown (Gotthardt et al., 2015). An UNC119 ortholog is encoded in the Chlamydomonas genome (Blast score 9e-52 over 96% of the sequence with human UNC119).*".*)*.

The mammalian ARL13/ARL3/UNC119 pathway was described in detail in the introduction. However, to my knowledge, UNC119 and other carriers have not been implicated in BBSome/IFT-dependent export of lipidated proteins from cilia. Further, PLD etc. are attached to the ciliary membrane and predicted to be dual lipidated whereas UNC119 etc. bind to monolipidated proteins to keep them soluble (=not attached to the membrane). After releasing them in cilia, UNC119 is believed to diffuse from the cilia back to the cell body; IFT or the BBSome have, to my knowledge, not been implicated in UNC119 export. I think it even is unknown if UNC119 moves by IFT at all. As this study focuses on the role of ARL13 in IFT/BBSome-dependent protein export from cilia, analyzing the role of UNC119 is out of the scope of this manuscript.

Overall response to the referee's concerns: The key observation of this paper is that IFT of PLD, which in control cells in BBSome dependent, is abolished in the *arl13* mutant. This clearly indicates that ARL13, directly or indirectly, is involved in protein export by the IFT/BBSome system, in addition to its role in ciliary protein import. If generally applicable, this observation could explain both the accumulation of hedgehog proteins in mammalian Arl13-/- cilia and the more severe phenotypes of *arl13* over *bbs* mutants. We further show that ARL13 levels and its

phosphorylation pattern are affected by BBS and IFT deficiency. We think that these are important findings, which connect the regulation of ciliary protein import with that of export.

Minor points
1. There are two Table S1 (page 26 and page 39). Probably, that on page 39 is Table 1.
2. Typographical errors:
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The mistakes were corrected.

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In this manuscript Dai et al. use Chlamydomonas as a model organism to study the function of ARL13 in ciliary membrane protein composition and transport. The vertebrate ARL13 homolog, ARL13B, has been well studied by several groups and mutations in ARL13B has been linked to defective Hedgehog signaling and ciliopathies in human and mouse.
Here the authors characterize a Chlamydomonas null mutant in ARL13 (arl13) and show using biochemical approaches (cilia isolation/fractionation, mass spectrometry, western blotting) that ARL13 loss leads to abnormal ciliary accumulation of some membrane proteins (e.g. PLD), and reduced ciliary levels of others (e.g. FAP12). They also show that endogenous and tagged ARL13 is localized in the ciliary membrane fraction, and occasionally/rarely moves by IFT, as observed before. In previous work the authors nicely showed that PLD is a cargo for the BBSome during ciliary protein export, hence they decided to focus here on the possible connection between ARL13, PLD and the BBSome. By using various Chlamydomonas mutant and rescue lines, combined with biochemical analysis, live cell/TIRF imaging and phenotype analysis (phototaxis assays), the authors provide evidence to suggest that ARL13 is required for mediating PLD association with the BBSome for its ciliary export, in turn affecting phototaxis. Loss of ARL13 seems not to grossly affect IFT/BBSome transport itself.

While the topic and results should be of great interest to cell- and developmental biologists, and the paper in principle would be suitable for JCB, I have several important concerns that need to be addressed by the authors before this manuscript is suitable for publication. Particularly, I find that lack of quantitative analysis of key western blot data (and some other data as well) is problematic, as it means that some of the main conclusions drawn by the authors are not fully justified by the data. Furthermore, some of the phenotype- and (partial) rescue data provided are confusing and require clarification. My specific comments are listed below.

We thank the referee for the supportive comments on our manuscript.

Main comments:

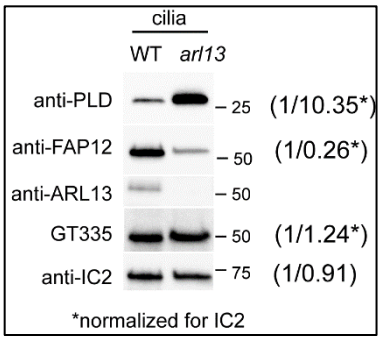
1) Issues with western blot data and interpretation thereof:

A substantial fraction of the figures includes western blot data comparing ciliary levels of various relevant proteins, in wild type and different mutant and rescue lines (e.g. Figures 1B, 1F, 2A, 3A, 3B4B, 4D5A, 5B, S1C, S1D, S2A-E, S3A, S3B, S5B). Based on visual inspection of these blots, the authors draw several (major) conclusions regarding relative ciliary levels of different proteins in the various strains. However, for some of the blots mentioned, the interpretation of the relative band intensities is not as obvious as postulated by the authors, especially because the loading is sometimes uneven, and no quantification of band intensities is provided.

We now provide quantifications for the key Western blots. If considered necessary, we are happy to quantify the other blots as well.

For example, in Figure S2B it looks like GT335 might be decreased in arl13 mutant cilia (considering the elevated IC2 signal), which is in direct contrast to what the authors write on bottom of page 8, but which would be in line with that reported by others in e.g. C. elegans.

The WB in question has been repeated with independent samples (Fig. S2E). Whereas the previous blot indeed showed a slight reduction of the GT335 signal (minus 26% compared to the control), the new blot shows a slight increase of the GT335 signal (plus 24%). Thus, the levels of polyglutamylated tubulin are not severely altered in *Chlamydomonas arl13* cilia as assessed using GT335 antibodies.



We revised the discussion of this point in the results section as follows: *"Potentially, these differences could be related to differences in cilia age: primary cilia of differentiated cells are typically long-lasting whereas Chlamydomonas disassembles its cilia day-to-day during cell division. These differences in cilia biology could also explain why ARL13b deficiency causes axonemal defects in C. elegans and mammalian cilia whereas those of the Chlamydomonas mutant were apparently normal (Caspary et al., 2007; Cevik et al., 2010; Li et al., 2010; Warburton-Pitt et al., 2014)."*

Another example is Figure S3A where it is hard to appreciate the increase in PLD levels in arl13 from 40-120 min (given reduced IC2 signal at 40 min), whereas Arl13 levels in the bbs4-1 mutant looks reduced initially (40 min) and then gradually increases.

The referee's impression is correct. The blot (now Fig. S3B) has been replaced with one showing more equal loading and a quantification has been provided. Also, we partially repeated

the experiment with essentially the same outcome, i.e., that PLD accumulates over time in regenerating *arl13* cilia.

The latter result suggests that the BBSome might be needed to keep ciliary levels of ARL13 low, which agrees with the blot in Fig. S3D. On the other hand, the increased ciliary ARL13 levels seen in the bbs4-1 mutant is apparently not rescued by BBS4-GFP (Figure 3B). (see below for response to this point) The authors comment on this on page 15, but the results are quite confusing and moreover, blots from the different strains shown in Figure S3A look to be from different gels (?) making direct comparison of band intensities nearly impossible for the reader.

The blot in Fig. S3B (formerly Fig. S3A) has been replaced with an uncut membrane to facilitate comparison of signal strength across strains.

On the other hand, the increased ciliary ARL13 levels seen in the bbs4-1 mutant is apparently not rescued by BBS4-GFP (Figure 3B).

The referee raises an interesting point, which we feel we have not considered fully in the previous version. Apparently, maintaining normal ARL13 levels requires more BBSomes than maintaining normal (=low) PLD levels. First, we now acknowledge this observation explicitly ("*Interestingly, cilia of the parental bbs4-1 BBS4-GFP strain had normal amounts of PLD whereas ARL13 remained elevated (Fig. 3B).").* Further, we discuss these data and suggest (as one possibility) that the BBSome might limit ARL13 entry into cilia and that reduced amounts of BBS4/BBSome, while sufficient for PLD export, fail to properly regulate the amount of ARL13 in cilia (*"A small numbers of BBSome could be sufficient to ensure PLD export via IFT but insufficient to limit ARL13 influx into cilia."*). Of note, several previous studies suggest that the BBSome also participates in the regulation of protein entry into cilia (Berbari et al., 2008; Loktev and Jackson, 2013; Yu et al., 2020).

In Figure 3B it is hard to compare endogenous BBS4 levels with BBS4-GFP as different antibodies were used to probe for the two. In this blot IC2 band intensity also looks lower in WT than in other lanes, again making comparison difficult.

It is correct that the levels of BBS4-GFP and endogenous BBS4 can't be compared as they were obtained with different antibodies. As previously reported the expression level of BBS4-GFP is well below those of endogenous BBS4, but nevertheless rescues ciliary PLD levels and phototaxis (Lechtreck et al., 2009; Liu and Lechtreck, 2018). Why BBS4-GFP fails to also rescue ARL13 levels is unclear. As mention above, we now discuss the possibility that the BBSome negatively regulates ARL13 entry into cilia.

It is also correct that IC2 signal in the wild-type control indicates underloading of this sample. The ARL13 signal strength was quantified, normalized for the IC2 signal, and the data were added to the blot.

Finally, in Figure S1D it looks like there is ALR13 in the last lane, but authors claim on page 6 bottom there is not. In Figure S1D, the labeling above the lanes is also misaligned.
This point was also raised by referee 3. The sentence in question referred to the fact that most of the ARL6/BBS3 moves from the aqueous to the detergent phase when GTPgammaS is present during phase partitioning whereas ARL13 does not. We now provide (in the figure and in the corresponding section of the result) a quantification of the anti-ARL13 signal in the different fractions obtained in these experiments.
p. 7: "*After fractionation of isolated cilia using Triton X-114 phase partitioning and centrifugation, most Chlamydomonas ARL13 (~65%; n=2) was found together with the IFT-B protein IFT81 in the soluble aqueous phase representing the ciliary matrix while smaller amounts were present in the axonemal (~35%) and membrane (~2%) fractions (Fig. S1H).*"

There are similar issues with some (but not all) of the other western blot figures. In summary, the authors need to carefully quantify the most central western blot results and make sure their conclusions are justified by the data provided.
The key western blot data (reduction of FAP12 and accumulation of PLD in *arl13* mutant cilia) are based on multiple independent biological replicates (i.e., independent isolates of cilia from the strains). In Fig. S2 A and B, we now provide a meta-analysis of these experiments showing that PLD accumulates on average ~50x in *arl13* cilia (n=8 biological replicates) whereas FAP12 is reduced on average by ~75% (n=7 biological replicates).

2) Figure 3D: these results are a bit confusing, is the apparent difference between the two strains statistically significant?
Fig. 3D compares the IFT frequency of BBS4-GFP in the *bbs4-1* to the *bbs4-1 arl13* double mutant background. Yes, the differences in BBSome transport frequency are statistically significant and the t-test values have been added to the figure (they should have been there to begin with). Also, this reduction in frequency is now repeatedly acknowledged (see below).

Importantly, a reduction of the BBS4-GFP transport frequency in a transgenic strain developed to image the BBSome in vivo does not necessarily show that BBSome traffic is altered in the *arl13* mutant, as western blots document normal levels of several BBS proteins in the mutant (Fig. 3A).

Could the (apparent) reduced IFT frequency in the arl13 mutant be related to possibly altered tubulin polyglutamylation (see comment above to Figure S2B)?
As we show in Fig. 2, the frequency of IFT is normal in *arl13*. Kubo et al showed that reduced levels of polyglutamylation do not affect IFT frequency/velocity in Chlamydomonas (DOI:10.1091/mbc.E15-03-0182). Further, polyglutamylation is apparently normal in the *arl13* mutant cilia (see new WB above). How changes in the track (i.e., the axonemal doublet microtubules) could affect BBSome transport frequency without affecting that of IFT is unclear.

As mentioned above, we revised the discussion of the tubulin polyglutamylation data and provide a possible explanation for species-specific differences.

Related to this: page 10 bottom and page 11 lines 7-8, are the authors 100% sure IFT/BBSome traffic is normal in the arl13 mutant strain? It is not so obvious based on the data shown in Figure 3D.

The referee correctly points out that the IFT frequency of BBS4-GFP was reduced in the *arl13 bbs4 BBS4-GFP* vs. the *bbs4 BBS4-GFP* strain. The phrase in question was rewritten and we now simply state that:" *We conclude that BBSome assembly and traffic are mostly normal in arl13 but for the reduction in frequency observed for BBS4-GFP.*" Our data show 1) the normal presence of IFT and BBS proteins in *arl13* cilia, 2) the normal length of *arl13* cilia, 3) the normal velocity and frequency of IFT54-mS in *arl13* and 4) the normal processivity and velocity of tagged BBSomes by IFT (albeit at a somewhat reduced frequency). Thus, we do not think that the reduced transport of BBS4-GFP in a certain strain explains the complete lack of PLD transport and we added the following statement on page 12, bottom: "*We consider it unlikely that a reduced ITF frequency of BBS4-GFP observed in the transgenic strain used to monitor BBSome traffic in vivo (Fig. 3D), explains the complete absence of PLD transport by IFT in arl13.*"

3) Figure 4C and page 13, 5 lines from top: the phototaxis data are not easy for a non-expert to interpret, and without quantitative analysis, how can authors claim that the arl3 mutant phototaxed "less efficiently" than control cells?

This statement was based on the increased number of *arl3* cells that did not phototax to the edge of the well within 2 minutes, which is visible by the increased green level in the middle of the well in comparison to control/WT. As the arl3 mutant is only peripherally relevant to the manuscript and our co-authors have a forthcoming study focusing on Chlamydomonas ARL3 , the phrase in question has been modified to: "*Despite the accumulation of PLD, which we previously characterized as a negative regulator of Chlamydomonas phototaxis, arl3 phototaxed ~~albeit somewhat less efficiently than control cells~~ (Fig. 4C; (Liu and Lechtreck, 2018)).*"

4) Rescue experiments: it is unfortunate that the phototaxis phenotype of the arl13 mutant cells cannot be fully rescued by expression of ARL13-mNG (Figure 1D and Figure S1H), which according to the authors could be due to low level expression of the transgene compared to endogenous ARL13 (Fig. 1B and 4D). Since the ARL13F53A-NG rescue construct is expressed at higher level than the wild type version (Figure 4C), did authors try to do phototaxis assay with this strain?

The *arl13 ARL13(F53A)-NG* strain showed somewhat improved phototactic behavior (new Fig. S1F/G), which is mentioned in the results section of the revised manuscript: "*In the arl13 ARL13^{F53A}-NG strain phototactic behavior was partially restored, which could be related to the*

higher levels of ARL13^{F53A}-NG expression in comparison to the arl13 ARL13-NG rescue strain (Figs. S5C, 4D)."

I think that we openly discussed that we do not understand the failure to rescue *arl13* phototaxis (despite the reduction/normalization of PLD levels) or the fact that *arl3* can phototax (despite PLD accumulation). Clearly, phototaxis is more complicated than no/low PLD > ptx and PLD accumulated > no ptx.

Related to this and page 8 first paragraph, could the authors test if PLD export from cilia is delayed in the arl13 ARL13-mNG strain?
One could try to express tagged PLD (like PLD-mS) and determine whther PLD lingers longer *arl13 ARL13-NG* cilia compared to controls. Practically, I'm not optimistic that the experiment will produce reliable data because PLD levels are exceedingly low in both strains. The aim of this rather time-consuming experiments is to explain why we failed to rescue phototaxis in *arl13*, which could have many other reasons unrelated to PLD transport. The experiment is unlikely to contribute to the main question of how ARL13regulates ciliary FAP12 and PLD levels, both of which are fully rescued by ARL13-NG expression.

Minor comments:

Figure S1H: numbers are very small and hard to read.
In the revised figure, we used a large font for these numbers.

Page 6, 2 lines from bottom: the authors cannot use the term "quantitatively" when the western blot results in Fig. S1D were not quantified.
We rephrased the sentence as follows: "*Addition of the non-hydrolyzable GTP analog GTP-γ-S during during phase partitioning moved ARL6/BBS3 from the matrix to the membrane fraction whereas most ARL13 remained in the matrix fraction (Fig. S1I).*" The western blots related to these data were quantified.

Page 7 middle: where is the data for the MS analysis referred to here? Is it the same as the data shown in the Table S1 located on page 39? Please clarify.
Yes, all MS data are summarized in Table 1 (which we by mistake referred to as Table S1).

Page 8 line 2: "inversion" should be "Inversin" as this compartment refers to the proximal region of the cilium occupied by the Inversin protein.
The "auto-correct" typo was corrected.

Page 9, 5 lines from bottom: "causes on accumulation" should be "causes accumulation".

Done

In many places throughout the text, arl13 mutant cells or arl13 mutant cilia are referred to simply as "arl13". Please specify whether you mean arl13 mutant cells or cilia.
We corrected these statements during the revision. ("*arl13*" alone refers to the cells or strain)

Page 16 top: the statement "In arl13 mutants, PLD failed to bind to the BBSomes" is not justified by the data provided, the authors only show accumulation of PLD in arl13 mutant cilia, not that PLD cannot bind to BBSomes. To justify this statement, authors would need to perform IP experiments or similar.
We modified the phrase as follows*:"We conclude that ARL13 is in some way required for the transport of PLD by IFT/BBSome carriers."*

Further down on the same page: "BBSome composition and traffic were apparently normal in arl13" is also not fully justified, given the data shown in Figure 4D (but see comment to this figure above).
Agreed; see above for how we addressed this point..

Mislabeling of Tables:

Page 26-28 contain three tables that are labeled Table S1, S2 and S3, respectively. Page 39 also includes a table named Table S1 (i.e. there are two Table S1). Page 8 bottom refers to a "Table 1" but there is no such table included, and the same goes for "Table 3.2", "Table S3.3", "Table 3.3" mentioned on page 20 and "Table 3.4" mentioned on page 23.
We apologize for the confusion. The labeling of the tables was corrected.

Table S2: it would be more informative for the reader if this table had a bit more information about what the different primers were used for, rather than just the numbers (even though some explanation is provided in the methods section).
As acknowledged by the referee, we mentioned the primers by number in the description of the actual experiments in the Materials and Methods section. The purpose of the table is simply to provide the actual primer sequences.

Reference list:

Mariani et a. 2016 and Yan and Shen 2021 are incomplete.
Indeed. I tried to fix it but Endnote, set to JCB style, doesn't like it. I believe the JCB or another journal will add the doi and reformat the reference list.

We appreciate the very thorough work of this referee.

Reviewer #3 (Comments to the Authors (Required)):

In their manuscript, "CHLAMYDOMONAS ARL13 FACILITATES BBSOME-CARGO INTERACTION FOR PROTEIN EXPORT FROM CILIA " Dai et al. use a combination of genetic mutants, biochemical analysis and sophisticated live imaging to investigate the function of ARL13 in Chlamydamonas. In the context of previous work in other mutants, the authors conclude that "Chlamydomonas ARL13 enables BBSome-cargo interactions to ensure the export of signaling proteins from cilia." This is important work as it advances the field's understanding of what exactly ARL13 might do, which is currently not understood. Overall, the work is rigorous in its experimentation with appropriate controls and replicates. The significance of the work is underscored by the fact that it provides a plausible mechanism through which to explain some of the phenotypic overlap and distinctions among various ciliopathies, assuming that the function of ARL13 is conserved.

We thank the referee for this positive assessment of the manuscript.

There are several points that must be addressed:
1) Chlamydamonas is the work horse organism of ciliary biologists and I cannot say enough about how much biology we understand due to its study. Indeed, there is so much conservation from Chlamy to human that underlies my excitement about this work. That said, the authors must make clear the distinction of ARL13 from ARL13A (about which we know almost nothing) and ARL13B (about which we know slightly more than nothing). I recognize that conflating ARL13 and ARL13B in Chlamydomonas was initiated in the Wittinghofer work from 2015 where they designated Chlamydamonas ARL13 as ARL13B in clear conflict with any nomenclature guidelines. This is not simply semantic as there may well be distinct evolution of ARL13 function to ARL13A and 13B. This work helps lay the groundwork for testing CrARL13 function in additional organisms. (Note, worm ARL13 is referred to incorrectly as ARL13B in at least one place I saw)

We agree and replaced ARL13B and *arl13b* for Chlamydomonas with ARL13/*arl13* throughout the manuscript. In the present and revised manuscript, we acknowledged the presence of a single *ARL13* gene in *Chlamydomonas* in the Introduction by writing: "*Chlamydomonas* possesses only a single *ARL13* gene whereas the gene is duplicated in most metazoans, i.e., *Arl13a* and *Arl13b* (Kahn et al., 2008; Schlacht et al., 2013). *Chlamydomonas* ARL13 (527 residues) and human Arl13b (428 residues) and are reciprocal best hits with a score of 2e-56 in protein BLAST and possess 44% sequence identity in the N-terminal G-loop indicating that the two are orthologues whereas Arl13a deviates considerably."

With regard to *C. elegans* ARL-13b, we added that this organism possesses only one ARL13 ortholog. We prefer, however, to not alter the nomenclature established by the *C. elegans* community.

2) The Chlamy arl13 allele needs to be more clearly defined/explained. The authors treat it as a null but are rather vague about the exact data supporting that assertion. Where is the insertion relative to the G motifs? Are they all there? What data do the authors have to state in the legend of Figure 1, "We have no evidence that a residual truncated ARL13 is expressed."? It appears that the antigen for the antibody is at the C terminus of the protein so would not detect a N-terminal truncated protein. What's up?

The referee is correct that we failed to properly report and explain the nature of the *arl13* allele. Since we didn't look for the presence of a truncated protein, the phrase "*We have no evidence that a residual truncated ARL13 is expressed*" was misleading and a foolish thing to write in the first place; the phrase was deleted. The antibody used in the manuscript was raised against the C-ter portion of the protein encoded by exon 2 down-stream of the insertion site. We tried and failed to raise an antibody against the N-terminal region of ARL13. Thus, it is unclear if this mutant is a true arl13 null.

This is now described in the first paragraph of the results: "*The insertion was confirmed by PCR and is predicted to introduce a premature stop codon after 474 bp, which, if residual expression should occur, will lead to a severely truncated and likely non-functional ARL13 lacking parts of its P-loop GTPase domain and most of the α6 helix, which participates in the binding of ARL3 (Fig. 1A, S1A; (Gotthardt et al., 2015)).*"

This assessment is also based on consultations with two experts on ARL GTPases (Rick Kahn, Emory and Marek Eliáš, University of Ostrava). We also contemplated to address this question by RT-PCR. However, as the insertion is in the first exon, it will not be easy to distinguish cDNA from traces of contaminating genomic DNA (i.e., the PCR products will be identical). As our revision required numerous new experiments, we did not attempt this RT-PCR experiment (yet). If this is an essential point, we would be happy to give it a try.

3) Where does ARL13 localize in Chlamy? Does the ARL13-mNeon reflect endogenous protein localization? The authors have an antibody- where do they see it via IF? This addresses the broader issue of where ARL13 functions in Chlamy. The final conclusion in Chlamy is that ARL13 in the matrix helps attach membrane proteins onto the BBSome but is that where the authors see it? What about Chlamy in which the membrane is removed- any change? On the flip side, previous work from the Barr and Blaque labs showed ARL13B functions at the ciliary axoneme yet the authors detect no defects in the Cr arl13 mutant axonemes. These are examples of where the manuscript appears to use ARL13 and ARL13B similarities only when convenient. ARL13B is not detectable on mammalian cilia where the membrane is stripped off. The authors

should discuss the possible reasons for such differences and how it might impact the model they propose for mammalian ciliary traffic.

We tested the antibody to *Chlamydomonas* ARL13 in immunofluorescence of both methanol and detergent-extracted aldehyde fixed cells. Cilia of the *arl13* mutant and control cells were stained with similar intensity revealing that the antibody is unsuited for immunohistochemistry. We briefly mention this in the revised manuscript: "*Since our antibody against ARL13 failed in immunofluorescence experiments (not shown), we expressed Chlamydomonas ARL13 fused at its C-terminus to mNeonGreen (ARL13-NG) under the control of the native ARL13 flanking sequences in the arl13 mutant (Fig. S2C).*"

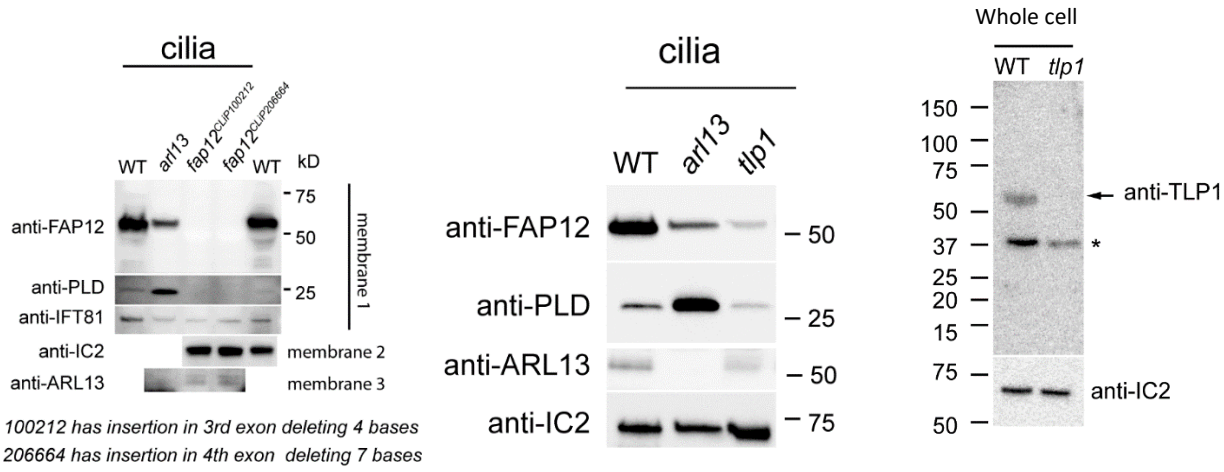
However, we think (and I believe the referee and the editors will agree) that the cilia fractionation experiments shown in Fig. S1 are much better suited to determine the sub-ciliary distribution of ARL13. The fractionation experiments show that most ARL13 is present in the soluble ciliary matrix fraction, which is in agreement with experiments showing that removal of the ciliary membrane solubilizes (most) ARL13. (p. 7/8: *"After fractionation of isolated cilia using Triton X-114 phase partitioning and centrifugation, most Chlamydomonas ARL13 (~65%; n=2) was found together with the IFT-B protein IFT81 in the soluble aqueous phase representing the ciliary matrix while smaller amounts were present in the axonemal (~35%) and membrane (~2%) fractions (Fig. S1H).*"). In the corresponding figure legend for S1, we state that the western blots indicate that the biochemical fractionation was incomplete as, for example, traces of IFT81 are still detected in the axonemal fraction.

To better reflect the similarities and differences between species, we wrote (p. 10): "*Potentially, these differences could be related to differences in cilia age: primary cilia of differentiated cells are typically long-lasting whereas Chlamydomonas disassembles its cilia day-to-day during cell division. These differences in cilia biology could also explain why ARL13b deficiency causes axonemal defects in C. elegans and mammalian cilia whereas those of the Chlamydomonas mutant were apparently normal (Caspary et al., 2007; Cevik et al., 2010; Li et al., 2010; Warburton-Pitt et al., 2014).*"

4) The authors do make a point at the end to acknowledge the role of ARL13 in ciliary protein entry as well as the model they put forth. Yet they use much stronger language in the abstract and manuscript. Rather than propose that "ARL13 enables BBSome-cargo interactions to ensure the export of signaling proteins from cilia", they conclude it. I very much like the model but it should be presented as a model. I think it is also possible that ARL13 could control ciliary entry of a factor X that is directly or indirectly responsible for the BBSome-cargo interactions. Obviously, any of the 4 proteins identified in the mass spec to be depleted in arl13 mutants would be prime candidates. Do the authors have data to refute such a possibility?
This critique is similar to objections from referee 1 and we agree. The title was changed ("*Chlamydomonas* ARL13 is required for BBSome-Cargo interaction during protein export from cilia") and the abstract was rewritten.

The referee is also correct that at this point we cannot exclude that ARL13 imports a "factor X" into cilia (or "activates" such a factor in cilia), which in turn is required for BBSome-PLD interaction. This is now explicitly and repeatedly stated in the manuscript.

Based on our analysis so far, the reduction of FAP12 is the main biochemical defects in *Chlamydomonas arl13* cilia. Thus, we obtained and analyzed two *fap12* mutants from the CLiP collection. These mutants apparently lack FAP12 but PLD levels (and those of ARL13 and BBS4, not shown) are normal. We also analyzed *tlp1*, a mutant in one of two Chlamydomonas TULP genes. The strain has severely reduced ciliary FAP12 levels but normal levels of PLD. While the data show that the reduction of FAP12 is not responsible to the accumulation of PLD, MS identified other proteins that are reduced/missing in *arl13* cilia. Thus, these data are too premature and incomplete (e.g., does FAP12 move by IFT?) to be reported in the current manuscript.



5) Along these lines, it is unclear whether there are literally only 4 enriched and 4 depleted proteins in the arl13 mutants or if the authors are only reporting 8 proteins and others are unreported. The authors should clarify.

Yes, that statement is meant literally. In the legend to the Table 1, we wrote *"Only proteins, which were enriched or reduced in all three replicates are shown."* While many other proteins were enriched/reduced in only one or two of the replicates, the table provides a comprehensive list of proteins altered significantly in all three MS replicates.

6) I don't think the authors are trying to say that the data supporting ARL13B as an ARL3 GEF is untrue on Page 13. Might be worth mentioning that the data would support there being additional ARL3 GEFs in vivo.

It is an established fact that ARL13 acts as a GEF for ARL3 in vitro. Here, we just report in vivo observations, which do not allow us to either support or dismiss ARL13 being a GEF for ARL3. We added "*Possibly, other cellular GEFs compensate for the loss of ARL13 GEF activity*." to the corresponding part of the results section.

7) Related to point 1, proper nomenclature should be used throughout- human genes are all capital letters in italics, mouse genes only capitalize first letter etc. This will help clarify what the authors are referring to. Adding clear designations on the proteins could help. For example, on page 7, I am interpreting the ARL13 fused to mNeon to be Chlamy Arl13. (and there's a future experiment to use mammalian ARL13A and ARL13B to see whether both rescue or whether distinct functions evolved to each).
We corrected the nomenclature and added "*Chlamydomonas*" to the phrase in question.

8) The references require detailed attention. Many are inaccurate. Some examples in regards to mammalian ARL13B include the Caspary 2007 reference describing work in mouse that did not connect the gene to Joubert syndrome and the Gigante 2020 manuscript that did not address the question of Arl13 in Chlamy much less show that there are 2 ARL13 proteins in metazoans. The correct references are needed. Additionally, there are papers on ARL13 in worm from the Barr lab showing that ARL13 plays critical roles in cilia structure and ciliary traffic- as well as the cited one from the Hu lab linking ARL13 to IFT. As the work presented here focus on ARL13, it is important the authors present the context accurately and discuss how the data might be reconciled (technical distinctions, species differences etc).
Working on ARL13 has been a learning process for us and we apologize for not correctly using the references and missing others entirely. We have corrected the references as suggested by referees 2 and 3.

We appreciate the constructive critiques of all three referees.

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June 13, 2022

RE: JCB Manuscript #202201050R

Dr. Karl Lechtreck
University of Georgia
Cellular Biology 120 Cedar Ave, 635 Biological Science Bldg
Athens, GA 30602

Dear Dr. Lechtreck,

Thank you for submitting your revised manuscript titled "CHLAMYDOMONAS ARL13 IS REQUIRED FOR BBSOME-CARGO INTERACTION DURING PROTEIN EXPORT FROM CILIA." Your manuscript has now been re-assessed by all three reviewers. We would be happy to publish your paper in JCB pending final revisions necessary to address remaining concerns of Reviewer #1 as well as to meet our formatting guidelines (see details below).

Please also address the following:
1- We strongly discourage the usage of bar graphs and instead recommend that you use violin plots.
2- As noted by Reviewer #1, the title needs to be softened. There is no biochemical evidence that ARL13B modulates the interaction of PLD with the BBSome. Please also discuss the possibility that PLD accumulation inside cilia may be part of a general ciliary stress response that is triggered by imbalances in ciliary trafficking.
3- In the same vein, the accumulation of ARL13B in bbs4 mutant flagella may be very indirect. This possibility needs to acknowledged.
4- The experiments with ARL13B[F53A] suggest that ARL3 does not function downstream of ARL13B in removing PLD from cilia. This is the most parsimonious interpretation and should be presented first.
5- Figure legends should indicate the exact name/ID of the strain used as 'WT.'
6- Typo on p. 21 'Similarly, PLD also accumulates in cilia of bbs mutants or when IFT is switched off because it requires moving IFT/BBS carriers to exit control cilia.'

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add scale bar to Figure S1F and a size marker to S1A. There are also several blot strips that indicate MW with upward pointing arrows, these panels should be adjusted to include the nearest MW marker in the image.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

6) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene,

ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or gifted to you by other investigators (and provide references where appropriate). Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary. If antibodies are not commercial please add a reference citation if possible.

7) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

8) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed. JCB format does not allow for supplementary references so please remove this section.

9) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures and 10 videos. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section. Please include one brief sentence per item.

10) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

11) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

12) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible. Source Data files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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

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

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

I think this revision has obscured the points that this paper should make.

First, the most important finding in this study is that arl13 mutation in Chlamydomonas caused a group of lipid-anchored membrane proteins, such as PLD, to accumulate in flagella/cilia, and another group of membrane proteins to fail to enter flagella/cilia. In addition, similar PLD accumulation was also seen in bbs4 and arl3 mutants.
I think there is no doubt about these facts and appreciate the previous findings by these authors that PLD accumulates in cilia of bbs mutants and that BBSome is involved in the export of PLD out of cilia (Lechtreck et al, 2009, JCB; and Lechtreck et al., 2013, JCB). However, it seems to me that the authors are forcing the PLD accumulation in arl13 mutant cilia to be linked to BBSome and ARL3 functions. I would like the authors to take advantage of the fact that PLD accumulates in the arl13 mutant cilia, but I think that this paper is not ready for publication in the JCB unless the following points are properly addressed.

Major points:
1. I think the only evidence in this paper linking ARL13 to BBSome function is the results in Figure 3C&D, which showed that anterograde and retrograde IFT frequency of BBS4-NG was subtly decreased in the arl13 mutant compared to control (How can a data set with such a large SD produce such a statistically significant difference? And, in this case, a violin or scatter plot would be better.). However, as this evidence alone does not directly link ARL13 to BBSome/IFT, the authors wrote in ABSTRACT of the revised manuscript, "We conclude that Chlamydomonas ARL13 - directly or indirectly - facilitates BBSome-cargo interactions to ensure protein export from cilia." and on page 13, "In conclusion, ARL13 somehow facilitates PLD transport by IFT/BBSome carriers." It seems unreasonable to take such a vague statement as a conclusion. Furthermore, TITLE of this paper (revised version) is "CHLAMYDOMONAS ARL13 IS REQUIRED FOR BBSOME-CARGO INTERACTION DURING PROTEIN EXPORT FROM CILIA". As I and another reviewer have pointed out before, can the authors really make this claim from the data

presented in this paper?
2. On the other hand, the authors showed that ARL13 was substantially accumulated within cilia in the bbs4 mutant, although this was not rescued by exogenous BBS4 expression (Fig. 3B), and that the phosphorylation state of ARL13 was changed in the bbs4 mutant (Figure 5A-D). Based on these data, the authors described in DISCUSSION (page 22, top), "we speculate that the BBSome and IFT could regulate ciliary ARL13 levels independently of each other."; that is, the authors suggested that ARL13 functions downstream of the BBSome. It seems incompatible with the above conclusion.
3. The authors described in DISCUSSION (page 21), "Further, PLD accumulates in Chlamydomonas arl3 cilia supporting the idea that ARL13 and ARL3 form a functional pair during the regulation of BBSome-dependent export of PLD as they apparently do in protein import." However, experiments using ARL13F53A do not support this speculation, and the authors stated the following (page 15), "Thus, two widely used models for ciliopathies do not recapitulate the grave effects of Arl13b mutations with diminished GEF activity on humans. Possibly, other cellular GEFs compensate for the loss of ARL13." In other words, the authors described that they cannot prove the possibility that ARL13 and ARL3 are functionally paired. These statements clearly contradict each other.

Minor points:
1. ABSTRACT: "If generally applicable, a dual role of ARL13 in ciliary protein import and BBSome-dependent export could explain the phenotypical overlap between Bardet-Biedl syndrome and the more severe Joubert syndrome." I think such speculation should be written in DISCUSSION and not in ABSTRACT.
2. Typographical errors
Page 12, bottom line, & page 17, penultimate line: ITF→IFT
Page 15, line 3 from the bottom: unknow → unknown
Figure 5C: anti-ARL13B→anti-ARL13

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

The authors have addressed all the issues I raised during initial review and I have no further comments. I recommend publication in JCB.

Reviewer #3 (Comments to the Authors (Required)):

The authors have satisfactorily addressed my previous concerns. Hopefully, the copy editor will follow the C. elegans and ARL nomenclature guidelines and not perpetuate previously published errors.

Response to the referee's and editors' suggestions

Please also address the following:
1- We strongly discourage the usage of bar graphs and instead recommend that you use violin plots.
Almost all bar graphs, including the one criticized by referee 1, have been replaced with violin plots. The sole exception is Fig. S2A, as this graph is based on low n.

2- As noted by Reviewer #1, the title needs to be softened. There is no biochemical evidence that ARL13B modulates the interaction of PLD with the BBSome.
The title was changed to: "LOSS OF ARL13 IMPEDES BBSOME-DEPENDENT CARGO EXPORT FROM *CHLAMYDOMONAS* CILIA".

Please also discuss the possibility that PLD accumulation inside cilia may be part of a general ciliary stress response that is triggered by imbalances in ciliary trafficking.
We think that this speculative idea is largely covered in the second part of the discussion, last sentence: *"Finally, ARL13 could generate conditions, e.g., indirectly via its role in protein import or as a GEF or GTPase, that ensure that PLD can be picked-up by the BBSome."*. To make this point cleared we added: "and ARL13 loss would then lead to a ciliary environment impeding BBSome-dependent export of PLD."

3- In the same vein, the accumulation of ARL13B in bbs4 mutant flagella may be very indirect. This possibility needs to acknowledged.
In the last part of the discussion, we now write: "*Thus, the BBSome, which is present at the ciliary base, could - directly or indirectly - limit ARL13 entry into cilia and low-frequency BBSome-independent IFT of ARL13 could be sufficient to balance this ARL13 influx;*

4- The experiments with ARL13B[F53A] suggest that ARL3 does not function downstream of ARL13B in removing PLD from cilia. This is the most parsimonious interpretation and should be presented first.

We agree and added the following phrase on p. 15: "*The most parsimonious interpretation is that Chlamydomonas ARL13 is not acting via its GEF activity through ARL3 to prevent PLD accumulation in cilia."*

5- Figure legends should indicate the exact name/ID of the strain used as 'WT.'
Strain g1 (Pazour et al., 1995) was used as a wild-type control in all experiments; the figure legends were modified accordingly. The reference was mentioned in the Materials and Methods section.

6- Typo on p. 21 'Similarly, PLD also accumulates in cilia of bbs mutants or when IFT is switched off because it requires moving IFT/BBS carriers to exit control cilia.'
? the phrase seems alright to me? We revised it as follows:
"Similarly, PLD also accumulates in cilia of bbs mutants and, because it requires moving IFT/BBS carriers to exit cilia, when IFT is switched off (Liu and Lechtreck, 2018)."

We also introduced a movie showing IFT of ARL13(F53A)-NG and a new panel to figure S3 (S3C) documenting rapid entry of ARL13-NG into *arl13*-derived cilia of *arl13* x *arl13 ARL13*-NG zygotes. This strengthens the point that PLD entry in *arl13* and *bbs4* cilia and exit during repair in zygotes occur with similar kinetics.

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

I think this revision has obscured the points that this paper should make.

First, the most important finding in this study is that arl13 mutation in Chlamydomonas caused a group of lipid-anchored membrane proteins, such as PLD, to accumulate in flagella/cilia, and another group of membrane proteins to fail to enter flagella/cilia. In addition, similar PLD accumulation was also seen in bbs4 and arl3 mutants.
I think there is no doubt about these facts and appreciate the previous findings by these authors that PLD accumulates in cilia of bbs mutants and that BBSome is involved in the export of PLD out of cilia (Lechtreck et al, 2009, JCB; and Lechtreck et al., 2013, JCB). However, it seems to me that the authors are forcing the PLD accumulation in arl13 mutant cilia to be linked to BBSome and ARL3 functions (Considering that we directly see that tagged PLD moves by IFT/BBS in controls but not in the *arl13* mutant, we do not agree with the referee. Is the reviewer suggesting that PLD accumulation and its failure to move by IFT in the arl13 mutant are not related to ARL13 and BBSome function? Whether this defect is caused directly or indirectly by the absence of ARL13 needs to be addressed in a separate study). I would like the authors to take advantage of the fact that PLD accumulates in the arl13 mutant cilia, but I think that this paper is not ready for publication in the JCB unless the following points are properly addressed.

Major points:
1. I think the only evidence in this paper linking ARL13 to BBSome function is the results in Figure 3C&D, which showed that anterograde and retrograde IFT frequency of BBS4-NG was subtly decreased in the arl13 mutant compared to control (How can a data set with such a large SD produce such a statistically significant difference? And, in this case, a violin or scatter plot would be better.).
The key data linking ARL13 and the BBSome are the failure of BBSome-dependent PLD traffic in *arl13* and the accumulation and abnormal phosphorylation pattern of ARL13 in *bbs* mutants.

The bar graph was replaced with a violin plot. We do not know why the transport frequency of BBS4-GFP varies so much between the individual cells analyzed in each experiment. However, this variation is similar to our previous observations on cargo transport frequency.

The data in Fig. 3C and D are based on a transgenic strains and other data suggest normal BBS proteins levels in the *arl13* mutant. As outlined in our response during the first revision, we doubt that the differences in BBS4-GFP transport frequency between control and *arl13*, while statistically significant, are biologically important.

However, as this evidence alone does not directly link ARL13 to BBSome/IFT, the authors wrote in ABSTRACT of the revised manuscript, "We conclude that Chlamydomonas ARL13 - directly

or indirectly - facilitates BBSome-cargo interactions to ensure protein export from cilia." and on page 13, "In conclusion, ARL13 somehow facilitates PLD transport by IFT/BBSome carriers." It seems unreasonable to take such a vague statement as a conclusion. Furthermore, TITLE of this paper (revised version) is "CHLAMYDOMONAS ARL13 IS REQUIRED FOR BBSOME-CARGO INTERACTION DURING PROTEIN EXPORT FROM CILIA". As I and another reviewer have pointed out before, can the authors really make this claim from the data presented in this paper?

We revised the title: LOSS OF ARL13 IMPEDES BBSOME-DEPENDENT CARGO EXPORT FROM CHLAMYDOMONAS CILIA.

The criticized phrase "In conclusion, ARL13 somehow facilitates PLD transport by IFT/BBSome carriers." Was replaced as follows: "*In conclusion, loss of ARL13 incapacitates PLD transport by apparently normal IFT/BBSome carriers*."

We also replaced the last two sentences of Abstract (i.e., the phase ""We conclude that Chlamydomonas ARL13 - directly or indirectly - facilitates BBSome-cargo interactions to ensure protein export from cilia." was deleted.)

An accumulation of proteins, i.e., of Smo and GPR161, in cilia of *arl13b* mutants/knock downs has previously been noted in other systems, but an explanation for this phenomenon is missing. Here, we provide convincing evidence that in *Chlamydomonas,* the accumulation of proteins in *arl13* cilia in caused by an incapacitation of the BBSome export pathway. Thus, ARL13 is required for the BBSome to do its job. Whether it does that directly or indirectly needs to be addressed in a separate study.

2. On the other hand, the authors showed that ARL13 was substantially accumulated within cilia in the bbs4 mutant, although this was not rescued by exogenous BBS4 expression (Fig. 3B), and that the phosphorylation state of ARL13 was changed in the bbs4 mutant (Figure 5A-D). Based on these data, the authors described in DISCUSSION (page 22, top), "we speculate that the BBSome and IFT could regulate ciliary ARL13 levels independently of each other."; that is, the authors suggested that ARL13 functions downstream of the BBSome. It seems incompatible with the above conclusion.

We disagree that these statements are incompatible. The data show that the BBSome can't export PLD in *arl13* and that ARL13 accumulates in *bbs* mutants. Thus, there is cross-talk between the two transport systems i.e., ciliary import and export. We elaborated on this idea in the last paragraph of the discussion.

3. The authors described in DISCUSSION (page 21), "Further, PLD accumulates in Chlamydomonas arl3 cilia supporting the idea that ARL13 and ARL3 form a functional pair during the regulation of BBSome-dependent export of PLD as they apparently do in protein import." However, experiments using ARL13F53A do not support this speculation, and the authors stated the following (page 15), "Thus, two widely used models for ciliopathies do not recapitulate the grave effects of Arl13b mutations with diminished GEF activity on humans. Possibly, other cellular GEFs compensate for the loss of ARL13." In other words, the authors described that they cannot prove the possibility that ARL13 and ARL3 are functionally paired. These statements clearly contradict each other.

We agree and revised the paragraph in question as described above.

Minor points:

1. ABSTRACT: "If generally applicable, a dual role of ARL13 in ciliary protein import and BBSome-dependent export could explain the phenotypical overlap between Bardet-Biedl syndrome and the more severe Joubert syndrome." I think such speculation should be written in DISCUSSION and not in ABSTRACT.
The phrase in question was removed from the abstract.

2. Typographical errors
Page 12, bottom line, & page 17, penultimate line: ITF→IFT
done
Page 15, line 3 from the bottom: unknow → unknown
done
Figure 5C: anti-ARL13B→anti-ARL13
done

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