

The human ciliopathy protein RSG1 links the CPLANE complex to transition zone architecture

Corresponding Author: Dr John Wallingford

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript started with three OFD patients who have mis-sense mutations in CPLANE2/RSG1. The authors first used *Xenopus* to show that these mutations disrupt Rsg1 function. They then identified GTP-dependent interaction between Rsg1 and transition zone protein Fam92A. They then used RSG1 KO human, and *Intu* and *Fuz* mouse cells to show that CPLANE proteins are important for transition zone protein localization. This work provided the last missing link in the CPLANE complex/human ciliopathy connection. It also identified a new connection between this complex and the ciliary transition zone. It should be of interest to the readers of Nature Communications.

Major point:

1. I was surprised that the authors did not provide any biochemical (in vitro interaction or co-IP, etc) or cell biological data (FRET, BiFC etc) to substantiate the direct interaction between Rsg1 and Fam92A. I feel this line of evidence would provide strong support for the model.

Minor points.

1. The authors should be consistent in their use of the gene names. Are the RSG KO cells in Fig 7 the same as the CPLANE2 KO cells in Fig 8? If so, please change one of them. I would suggest using the term RSG1 to cause less confusion as similar terms have been used throughout the manuscript.
2. On top of page 8, a sentence reads "Even stronger scores were found when we modeled and RSG1 monomer with only the C-terminus of FAM92A". Please check this sentence as there seems to be a typo here.

Reviewer #2

(Remarks to the Author)

This study identifies RSG1 (CPLANE2) as causative in human ciliopathy, specifically linked to phenotypes characteristic of Oral-Facial-Digital Syndrome (OFD), and investigates the mechanism of disease in frog and mammalian ciliated cells. These findings are supportive of RSG1 knockout mice studies which show ciliopathy-related developmental abnormalities. The study reveals patient variants affect critical steps in ciliogenesis, including basal body docking and the recruitment of intraflagellar transport (IFT) proteins in *Xenopus* embryos. Using AlphaFold3, the researchers predict that RSG1 can directly bind the transition zone protein FAM92. This interaction is validated through affinity purification and mass spectrometry, demonstrating a GTP-dependence. The authors extend their findings to show that RSG1 is involved in the recruitment of FAM92 and other transition zone proteins to the basal body. They also highlight a shared disease mechanism with other CPLANE complex proteins, in relation to defects in basal body docking and transition zone protein recruitment. Overall this work is of a high degree of significance as it provides new mechanistic insight into CPLANE normal and disease function in particular as it pertains to assembly of the ciliary transition zone assembly, which remains poorly understood.

It would be beneficial for the authors to expand on the dysfunction of motile multiciliated cells (MCCs) and its connection to patient disease in the main text, given their use of *Xenopus* MCCs to investigate Rsg1 and patient variants. Additionally, considering the more direct link between primary cilia and disease, it would be interesting to know if the patient variants can rescue ciliogenesis and transition zone (TZ) recruitment in RSG1 knockout cells. Conducting biochemical studies to evaluate if FAM92 association is disrupted by RSG1 variants may provide enhanced disease mechanism insight, although I

appreciate variant expression levels may be limiting. The authors note G118 resides in the GTP binding pocket, so understanding its impact on the GTPase-dependent functionality of RSG1 could be informative. Furthermore, it would be interesting to explore whether the interactions of other patient variants are similarly affected to further connect patient disease causes.

In relation to the preceding comment, RSG1 interaction with FAM92 is predicted by AlphaFold and identified by APMS, although experimental biochemical evidence could be strengthened by biochemical direct binding or minimally by Co-IP analysis.

Considering the transition zone proteins are affected in RPE1 cells might Arl13b ciliary localization may also be predicted to be affected. This could be ruled out by checking ciliation impairment using an axonemal marker such as Actub.

Given the basal body docking issue observed in RSG1 depletion have the authors considered whether RSG1 could function in basal body maturation? For example, are transition fibers present?

To strengthen the connection to GFP-IFT43 basal body localization data in Figure 3 an immunoblot demonstrating this protein is expressed comparably in embryos with Rsg1 wt and variants should be considered. This would demonstrate if patient variants affected IFTA expression/stability.

Patient variant expression data needs some clarification in the text and possibly quantification related to Figure 2. The authors note that normal expression is observed for D184W, however from the blot it looks like the protein levels are lower than the wt. Is there any concern that the amino acid charge difference between human and frog corresponding to 184 could affect expression/function? Suggest removing the statement about 'enrichment of G114E in cytoplasm' as data is not shown and the western blotting confirms expression is affected compared to wt.

Minor points:

The APMS of RSG1 is described in the main text as being performed in IMCD3 cells. However, in the methods section, it is mentioned as being performed in RPE1 cells. A description of how IMCD3 cells were generated should also be included if these cell line have not been previously described.

In Figure 7, the FAM92A signal is still detectable, though weaker, in RSG1 knockout (KO) cells compared to wild-type (WT) cells. This would seem to suggest that some FAM92A is recruited through an RSG1-independent pathway. I think it is important to add a comment about this possibility and/or an alternative explanation for this result in the discussion.

For Figures 3 and 4 is the expression of variants similar in MO treatments to what is shown in Sup Fig S2. Could the presence of the endogenous Rsg1 affect variant expression?

Are basal body numbers affected in Rsg1 KD and rescue studies. If not more representative images should be considered for Figure 4. It may be that different sized cells or cell orientations are shown which give the appearance of differences in these structures.

Figure 7 and 8 could be combined since both figures examine transition zone protein recruitment by RSG1.

Page 4. Typo. 'Mapping variant residues onto the predicted structure revealed that and A76 anchors the $\alpha 1$ helix' change to 'Mapping variant residues onto the predicted structure revealed that A76 anchors the $\alpha 1$ helix'

Reviewer #3

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The manuscript by Neftali et al. 'The human ciliopathy protein RSG1 links the CPLANE complex to transition zone architecture' extends our knowledge of another but understudied ciliary complex involved in ciliary trafficking, both with the first human disease reports for RSG1/CPLANE2 and functionally with interaction studies, computational modelling and wet lab hypothesis testing. It represents a significant body of work which will be of great interest to many readers in the cell biology, developmental biology and human genetics fields. However, there are several significant issues that must be addressed before publication in Nature Communications, in terms of accuracy and clarity.

Major issues

1. Human Genetics needs better structure/information. Are these the first clinical reports of these variants? If yes, much more care and effort must be put into sharing this data in a clear and concise manner. The current table is a data dump and very difficult to track. Norms would be a separate clinical features table summarizing defects (similarities some hierarchy: congenital heart defects? Palate defects?) and a genetics table where you capture the change in the genome, the cDNA and the protein, the zygosity, inheritance mode, platform used, singleton/trio sequencing and criteria for the ACMG that was met to allow the molecular diagnosis to be made. I would suggest they also include PolyPhen2, Grantham, SIFT scores etc- something about how these variants are predicted to affect protein.

This is an issue as 2/3 pathogenic variants do not affect conserved residues- the first A76 altered in chicken and *Xenopus*, and the third R188 and least compelling link is not conserved in any organisms outside mouse (zebrafish, fugu, cow, pig, chicken, *xenopus* etc). More concerning is that in gnomAD (supposedly healthy population) there are two individuals with homozygous changes in the same residue R188Q change to glutamine. It is a R188W SNP found heavily in various sequencing cohorts as heterozygous and predictions suggest unlikely pathogenic. See screengrab. It is important that the bar for this is high- if we are to make a molecular diagnosis for these patients. In contrast, G118 is extremely highly conserved across all and the variant is predicted to be pathogenic.

Pedigrees should show if unaffected sibs were tested. Details of any other biallelic pathogenic variants in other genes should be mentioned and how they were ruled in/out. Currently there is a random statement in the discussion that should not be there 'These OFD-affected individuals showed no other variants in genes previously associated with this condition after exome sequencing'. Confirmation of exome sequencing by Sanger- were molecular diagnoses issued to the families?

2. Pathogenic testing of RSG1 variants. This comes back to the first point that there is a lack of clarity on what Protein IDs were used for the alignments in Supp Fig 3. In Ensembl, the *Xenopus tropicalis* protein ENSXETP00000081015 has a different N- terminal and alignment to what is shown here only 57% identical. What does (l) and (s) mean? Why were these limited vertebrate species chosen as paralogues? Does changing a single amino acid, when not conserved and in a more divergent paralogue not create a Frankenstein protein, more distant to what was being assayed? Can human wild type RGS1 rescue morphant Rgs1 frogs? Were patient cell lines not available to test this directly? Especially in Sup Figure 4 only shows a single immunoblot and loading control of the samples. Reduced intensity of this variant compared to control or other variants is not an indication of stability as it could be injection efficiency, mRNA stability, translation, protein decay? Without more controls Sup Figure 4 only shows that the GFP-fusion protein is present, the reason for relatively reduced remains to be investigated. Some other type of validation is needed for the R188 variant, given all of the above.

3. Basal body docking. 3D projection Images in Figure 4, show a significant number of BB below the apical surface in RSG1 KO MCCs, this is used as an indication of the role of CPLANE on basal body docking. However, IFT43-GFP intensities, which are used as proxy of phenotypic rescue are done on a single plane where most centrin-RFP BB are found for a small number of cells. Without indications of which plane was selected, it is not clear if these are the ones at the most apical position, those migrating towards or those at the most basal. Is there a spatial gradient of GFP intensities along these planes? Do all cells show same rescue pattern?

4. Are embryos used for Figure 3 and 4 the same? More information is needed, the only Rsg1 variants listed are GFP in the Methods, but clearly IFT43-GFP over-expression would preclude this analysis? Figure 4 would benefit from two colour images. Are the migrating BBs already associated with CPLANE proteins? This could provide important mechanistic insight to the manuscript and highlight a functional difference with primary cilia where RSG1 is found in the mother centriole (Agbu et al. JCB 2018), bearing in mind that migrating centrioles are not a feature of RPE1 cells. And is particularly relevant for the working hypothesis that reduced BB docking is part of the pathogenic phenotype of RSG1 patients.

5. The two colour display (Centrin-RFP / RSG1-GFP) is also important for comparison with previously published findings (Grey, Nat Cell Biol 2009) where Centrin-RFP is confined to the apically docked centrioles while CLAMP-GFP are abnormally migrating complexes in Fuz morphants (panel b"), also observed in RSG1 Mo (panel d). However, the manuscript under revision shows the same abnormal migration now with Centrin-RFP. What has changed? Do we have two types of migrating BBs ones that are Centrin positive and ones that only contain CPLANE or associated components? Further, in the previous publication RSG1 MO completely eliminated the accumulation of CLAMP-GFP to the apical surface, whereas the manuscript under revision presents only partial abrogation of Centrin-RFP apical docking. Again raising the question, are CLAMP and Centrin migrating BB different in any way?

6. Migrating centrioles are a normal process of MCCs differentiation expected to be found in WT embryos as well? Would these immature cells have less, same or a gradient of IFT-43 GFP as well? Should a comparison be made between immature cells where both WT, mutants and rescues are done in cells with migrating centrioles?

7. Mass spec negative control. These results can only display what is different between both pull downs but not what is specific as the negative control is absent. PRIDE links were not shared so data could not be evaluated in review.

8. Dominant negative hypothesis. Although slightly tangential to the patient variants being examined, this APMS experiment is super important and completely not discussed. The RSG1-T69N mutant was generated to create a purported Dominant Negative effect, mentioned again here. If like Ras-S17N (Nassar et al., 2010) RSG1-T69N would sequester upstream activators and would be unable to activate downstream effectors. Regarding this hypothesis (INTU/Fuzz putative RSG1 GEF), then RSG1-T69N would bind INTU/Fuzz, with higher affinity than wild-type RSG1, and would localise to the ciliary base (but this does not seem the case (Agbu et al., 2018)). Moreover the APMS data here fully supports an alternative model.

So it appears RSG1-T69N does not have a dominant negative effect. But RSG1-T69N would be different from the Ras-S17N mutant. RSG1-T69N is probably not in a "GDP-locked" state (Lee et al., 2009; Bos et al., 2007). Asparagine at position 69 would prevent RSG1 binding of Magnesium and would also interfere with GDP/GTP binding. Regarding this hypothesis (INTU/Fuzz putative RSG1 GEF), then RSG1-T69N would be unable to bind INTU/Fuzz putative GEF complex, and then would be unable to localise to the ciliary base (which seems to be the case (Agbu et al., 2018))

9. Data not shown- either you show the data (Supplemental) or delete all sections with these statements in relation to Fam92A? Does wild type FAM92 localize to TZ BB in Xenopus and how is it changed in Rsg1 KDs? Given IFTA is known to scavenge TZ proteins in axoneme- could the phenotype be a secondary? CEP192 is a centrosome marker- how to distinguish from all centrosomes versus those to become basal bodies and fail- say CEP164? Something to mark mother centriole? For example, Fig. 8G- how do you know what a transition zone is in a non-ciliated cell?

10. Nomenclature throughout. Throughout all the text, figure legends and figures- it is chaos. Human genes upper case italics, human proteins upper case. Mouse genes title case italics, mouse proteins upper case. Xenopus genes appear lower case italics, and proteins title case. It is extremely confusing to readers.

Minor issues

1. Missing reference on discussion at top of page 7 on hexa-longin GEF complexes: DOI: 10.1093/bioinformatics/btz739
2. Details of what is being quantified in most graphs? What is each data point- a single centriole (from how many cells, from how many embryos, from how many injection runs) Ideally plotted as super-plots so that means of individual animals and experimental runs can be separated to understand origin of variation. Similarly in the methods, there is a statement which needs clarifying to be informative- 'Each experiment includes multiple biological replicates, conducted on different days.' Biological replicate- different embryos always same injection mix?
3. Plasmids used- no reference to nonGFP-tagged constructs- how to validate expression (localization/function etc)?
4. Lacking data of LAP-tag constructs- what was tagged (human, mouse?), where and single copy/polyclonal etc. Methods say RPE1 and text says IMCD3 cells?
5. Lacking details of MEFs used- primary? Immortalized? From what stage embryo? Reference in Methods too please.
6. Inaccessible proteomics dataset- not publically released and no reviewer key provided. Journal editorial desks should check this ahead of sending out to review to allow datasets to be properly interrogated.

Typos/Edits

1. Tone down the organism 'Top Trumps'- 'Xenopus, a proven platform for modelling the cell biology of ciliopathies'. Similarly the third paragraph of the introduction, emphasis on species dilutes the understanding of the molecular pathway. OFD is not a conventional motile ciliopathy- why jump to this motile, multiciliated cell type, versus say patient fibroblasts or modelling in human cell line?
2. p.4 3rd paragraph- delete 'that results in a Arg188Trp change in the coding region'- redundant.
3. In the text, the third patient p.ARG188TRP is supposed to relate to Supp Fig 1 but this patient in the text is not supposed to have polydactyly? Misreference- Supp Fig Legend refers to patient 2.
4. Typo- delete and 'structure revealed that and A76 anchors the a1'
5. Missing word- if suspected ciliopathy alleles of CPLANE2/RSG1 did likewise.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed all my concerns, and I now support the publication of this manuscript.

Reviewer #2

(Remarks to the Author)

The authors have significantly improved the manuscript with new supporting studies and analysis.

Two follow up points I wanted to make.

I appreciate the authors agreeing with my comment in regards to Figure 3 (IFT-43 expression) that immunoblot analysis might be informative. It is not clear to me why this study is beyond the scope of the present work. I think this would have strengthened the conclusions and ruled out a different explanation for why IFT-A components showed reduced expression at the basal bodies. If the overall cellular fluorescence signal was not deemed to be noticeably different in Rsg1 wild-type and variants that satisfies my question.

With respect to the added biochemical characterization of Rsg1 and Fam92 shown in Supplemental Fig 6A I think it would be beneficial to show the direct biochemical evidence of this interaction in the main figure to support the predictions. Did the authors attempt to confirm the predicted interaction with the C-terminal domain of Fam92? This result would also validate the predictions.

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Response to reviewer comments.

We thank the Reviewers for their overall enthusiasm for this project and for their diligent consideration of the work. As you will see in the detailed response below, we have done our utmost to address all concerns.

Reviewer #1 (Remarks to the Author):

This manuscript started with three OFD patients who have mis-sense mutations in CPLANE2/RSG1. The authors first used *Xenopus* to show that these mutations disrupt Rsg1 function. They then identified GTP-dependent interaction between Rsg1 and transition zone protein Fam92A. They then used RSG1 KO human, and Intu and Fuz mouse cells to show that CPLANE proteins are important for transition zone protein localization. This work provided the last missing link in the CPLANE complex/human ciliopathy connection. It also identified a new connection between this complex and the ciliary transition zone. It should be of interest to the readers of Nature Communications.

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1. To strengthen our data as suggested, we have performed *in vitro* co-IP to confirm the direct interaction between Rsg1 and Fam92a. This can be found in Supp. Fig 6A.

Minor points.

1. The authors should be consistent in their use of the gene names. Are the RSG KO cells in Fig 7 the same as the CPLANE2 KO cells in Fig 8? If so, please change one of them. I would suggest using the term RSG1 to cause less confusion as similar terms have been used throughout the manuscript.

2. On top of page 8, a sentence reads “Even stronger scores were found when we modeled and RSG1 monomer with only the C-terminus of FAM92A”. Please check this sentence as there seems to be a typo here.

2. These have been corrected.

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This study identifies RSG1 (CPLANE2) as causative in human ciliopathy, specifically linked to phenotypes characteristic of Oral-Facial-Digital Syndrome (OFD), and investigates the mechanism of disease in frog and mammalian ciliated cells. These findings are supportive of RSG1 knockout mice studies which show ciliopathy-related developmental abnormalities. The study reveals patient variants affect critical steps in ciliogenesis, including basal body docking and the recruitment of intraflagellar transport (IFT) proteins in *Xenopus* embryos. Using AlphaFold3, the researchers predict that

RSG1 can directly bind the transition zone protein FAM92. This interaction is validated through affinity purification and mass spectrometry, demonstrating a GTP-dependence. The authors extend their findings to show that RSG1 is involved in the recruitment of FAM92 and other transition zone proteins to the basal body. They also highlight a shared disease mechanism with other CPLANE complex proteins, in relation to defects in basal body docking and transition zone protein recruitment. Overall this work is of a high degree of significance as it provides new mechanistic insight into CPLANE normal and disease function in particular as it pertains to assembly of the ciliary transition zone assembly, which remains poorly understood.

It would be beneficial for the authors to expand on the dysfunction of motile multiciliated cells (MCCs) and its connection to patient disease in the main text, given their use of *Xenopus* MCCs to investigate Rsg1 and patient variants. Additionally, considering the more direct link between primary cilia and disease, it would be interesting to know if the patient variants can rescue ciliogenesis and transition zone (TZ) recruitment in RSG1 knockout cells. Conducting biochemical studies to evaluate if FAM92 association is disrupted by RSG1 variants may provide enhanced disease mechanism insight, although I appreciate variant expression levels may be limiting. The authors note G118 resides in the GTP binding pocket, so understanding its impact on the GTPase-dependent functionality of RSG1 could be informative. Furthermore, it would be interesting to explore whether the interactions of other patient variants are similarly affected to further connect patient disease causes.

In relation to the preceding comment, RSG1 interaction with FAM92 is predicted by AlphaFold and identified by APMS, although experimental biochemical evidence could be strengthened by biochemical direct binding or minimally by Co-IP analysis.

3. Reviewer #1 had the same suggestion (Comment 1), and we have now done the co-IP. We have added this result to Supp. Fig. 6A.

Considering the transition zone proteins are affected in RPE1 cells might Arl13b ciliary localization may also be predicted to be affected. This could be ruled out by checking ciliation impairment using an axonemal marker such as Actub.

4. This is an important point, and we've added the proposed experiment with AcTub to Fig. 8G. Crucially, the results are the same with the new marker.

Given the basal body docking issue observed in RSG1 depletion have the authors considered whether RSG1 could function in basal body maturation? For example, are transition fibers present?

5. Another good question. We explored this issue in *Xenopus* MCCs, finding that while Fam92b is reduced, Cep162 is slightly elevated (Supp. Fig. 7A-C). This result is consistent with the accumulation of certain TZ proteins observed after loss of IFT-A (See comment 24, to Reviewer 3, below).

To strengthen the connection to GFP-IFT43 basal body localization data in Figure 3 an immunoblot demonstrating this protein is expressed comparably in embryos with Rsg1 wt and variants should be considered. This would demonstrate if patient variants affected IFTA expression/stability.

6. While we agree this experiment might be informative, we feel it is unlikely to change the conclusions of the paper. We hope that, given the substantial other new data added to the revision, the reviewer agrees it is beyond the scope of the present work.

Patient variant expression data needs some clarification in the text and possibly quantification related to Figure 2. The authors note that normal expression is observed for D184W, however from the blot it looks like the protein levels are lower than the wt. Is there any concern that the amino acid charge difference between human and frog corresponding to 184 could affect expression/function? Suggest removing the statement about 'enrichment of G114E in cytoplasm' as data is not shown and the western blotting confirms expression is affected compared to wt.

7. The statement has been removed.

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In Figure 7, the FAM92A signal is still detectable, though weaker, in RSG1 knockout (KO) cells compared to wild-type (WT) cells. This would seem to suggest that some FAM92A is recruited through an RSG1-independent pathway. I think it is important to add a comment about this possibility and/or an alternative explanation for this result in the discussion.

8. This is an interesting point. We therefore explored it further and found that *a)* loss of Fam92 is also partial after Rsg1 KD in frogs and *b)* Fam92 is present at low levels in BBs before docking in the cytoplasm of *Xenopus* MCCs but accumulates dramatically in docked basal bodies (New Fig. 7). Thus, it appears that an early Rsg1-independent mechanism recruits Fam92 to undocked BBs and late, Rsg1-dependent mechanism acts later to strongly enrich Fam92 at docked BBs.

We regret our oversight on the generation of IMCD3 cells being accidentally labeled RPE1. This has been corrected in the methods.

For Figures 3 and 4 is the expression of variants similar in MO treatments to what is shown in Sup Fig S2. Could the presence of the endogenous Rsg1 affect variant

expression?

Are basal body numbers affected in Rsg1 KD and rescue studies. If not more representative images should be considered for Figure 4. It may be that different sized cells or cell orientations are shown which give the appearance of differences in these structures.

9. In the control, basal bodies (BBs) are spread on a larger apical surface area than the cells with a docking phenotype, thus, a slice in a control condition will have less BBs than the BBs aggregating in a smaller apical area in the cells with a docking phenotype. This has been clarified.

Figure 7 and 8 could be combined since both figures examine transition zone protein recruitment by RSG1.

10. Figures 7 and 8 are now Figures 8 and 9. We disagree with this minor point due to the amount of data in each of the figures. With Figure 8 showing the Rsg1 KO effects on ciliation and Fam92a recruitment. While Figure 9 contains the KO effects of not just Rsg1 but multiple CPLANE subunits on Nphp1, a core TZ component.

Page 4. Typo. 'Mapping variant residues onto the predicted structure revealed that and A76 anchors the α 1 helix' change to 'Mapping variant residues onto the predicted structure revealed that A76 anchors the α 1 helix'

11. These minor points have been corrected.

Reviewer #3 (Remarks to the Author):

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inheritance mode, platform used, singleton/trio sequencing and criteria for the ACMG that was met to allow the molecular diagnosis to be made. I would suggest they also include PolyPhen2, Grantham, SIFT scores etc- something about how these variants are predicted to affect protein.

12. We regret the original manuscript was less than comprehensive. The revised MS now has two clinical tables, a more-complete table describing the clinical features and a new table reporting molecular information (i.e., genomic positions etc., and also SIFT/PolyPhen scores, etc.)

This is an issue as 2/3 pathogenic variants do not affect conserved residues- the first A76 altered in chicken and Xenopus, and the third R188 and least compelling link is not conserved in any organisms outside mouse (zebrafish, fugu, cow, pig, chicken. xenopus etc). More concerning is that in gnomAD (supposedly healthy population) there are two individuals with homozygous changes in the same residue R188Q change to glutamine. It is a R188W SNP found heavily in various sequencing cohorts as heterozygous and predictions suggest unlikely pathogenic. See screengrab. It is important that the bar for this is high- if we are to make a molecular diagnosis for these patients. In contrast, G118 is extremely highly conserved across all and the variant is predicted to be pathogenic.

13. This is a crucial point, and we regret that in our effort to streamline this very large paper, we neglected to elaborate on the evolutionary conservation of these residues. As we now explain in detail on page 4-5 of the revision, there is good evidence that function of all three residues is conserved between human and frog. Finally, we believe the reviewer is incorrect about R188W. It is in fact a very rare allele in gnomad and SIFT calls it as deleterious (even though PolyPhen says benign). All of this is now discussed in the revision.

Pedigrees should show if unaffected sibs were tested. Details of any other biallelic pathogenic variants in other genes should be mentioned and how they were ruled in/out. Currently there is a random statement in the discussion that should not be there 'These OFD-affected individuals showed no other variants in genes previously associated with this condition after exome sequencing'. Confirmation of exome sequencing by Sanger- were molecular diagnoses issued to the families?

14. Testing of unaffected sibs was not possible. In Switzerland (Patient 2), it is legally problematic (not allowed) to do genetic testing on healthy minors. In Family 3, it was not possible to obtain samples from the sisters given that the family moved out of the country. We have however clarified that all alleles were confirmed by Sanger Sequencing.

2. Pathogenic testing of RSG1 variants. This comes back to the first point that there is a lack of clarity on what Protein IDs were used for the alignments in Supp Fig 3. In Ensembl, the Xenopus tropicalis protein ENSXETP00000081015 has a different N-terminal and alignment to what is shown here only 57% identical. What does (l) and (s) mean? Why were these limited vertebrate species chosen as paralogues? Does

changing a single amino acid, when not conserved and in a more divergent paralogue not create a Frankenstein protein, more distant to what was being assayed?

15. Please see new discussion of conservation of residues on page 4-5 (as per point 9, above). L and S refer to the alloalleles of the allotetraploid *Xenopus laevis* genome. This has now been clarified.

Can human wild type RGS1 rescue morphant Rgs1 frogs? Were patient cell lines not available to test this directly? Especially in Sup Figure 4 only shows a single immunoblot and loading control of the samples. Reduced intensity of this variant compared to control or other variants is not an indication of stability as it could be injection efficiency, mRNA stability, translation, protein decay? Without more controls Sup Figure 4 only shows that the GFP-fusion protein is present, the reason for relatively reduced remains to be investigated. Some other type of validation is needed for the R188 variant, given all of the above.

16. Patient cell lines are not available, so we used *Xenopus* assays. That said, we concede that the reduction of the GFP-fusion protein may not reflect stability but could instead reflect translation efficiency or protein decay, and this is now noted in the revision. As for the R188 allele, we now discuss this allele in more depth (see pt. 9 above), as well as the possibility that our two assays in frog do not reflect pathogenicity in human.

3. Basal body docking. 3D projection Images in Figure 4, show a significant number of BB below the apical surface in RSG1 KO MCCs, this is used as an indication of the role of CPLANE on basal body docking. However, IFT43-GFP intensities, which are used as proxy of phenotypic rescue are done on a single plane where most centrin-RFP BB are found for a small number of cells. Without indications of which plane was selected, it is not clear if these are the ones at the most apical position, those migrating towards or those at the most basal. Is there a spatial gradient of GFP intensities along these planes? Do all cells show same rescue pattern?

17. We now indicate which imaging plane was used for which experiment. Moreover, as we now show in Supp. Fig. 7, Ift43 is present at docked BBs and undocked BBs at multiple depths.

4. Are embryos used for Figure 3 and 4 the same? More information is needed, the only Rsg1 variants listed are GFP in the Methods, but clearly IFT43-GFP over-expression would preclude this analysis? Figure 4 would benefit from two colour images.

18. Yes, the data for Figures 3 and 4 come from the same experiments. However, we have decided to display the two phenotypes -Ift43 localization and docking- separately to make distinct points. We chose to remove the green IFT43 channel from Figure 4 since that phenotype is more clearly shown in Figure 3. Rescue was performed with Flag tagged Rsg1 constructs; this has been clarified in the manuscript.

Are the migrating BBs already associated with CPLANE proteins? This could provide important mechanistic insight to the manuscript and highlight a functional difference with primary cilia where RSG1 is found in the mother centriole (Agbu et al. JCB 2018), bearing in mind that migrating centrioles are not a feature of RPE1 cells. And is particularly relevant for the working hypothesis that reduced BB docking is part of the pathogenic phenotype of RSG1 patients.

19. This is a great question, and one we also deal with in Point 6 for Reviewer 2, above. Briefly, in new Fig. 7, we now show the dynamics of Fam92 and Rsg1 association with docking BBs and we discuss how this relates to the findings of Agbu.

5. The two colour display (Centrin-RFP / RSG1-GFP) is also important for comparison with previously published findings (Grey, Nat Cell Biol 2009) where Centrin-RFP is confined to the apically docked centrioles while CLAMP-GFP are abnormally migrating complexes in Fuz morphants (panel b”), also observed in RSG1 Mo (panel d). However, the manuscript under revision shows the same abnormal migration now with Centrin-RFP. What has changed? Do we have two types of migrating BBs ones that are Centrin positive and ones that only contain CPLANE or associated components? Further, in the previous publication RSG1 MO completely eliminated the accumulation of CLAMP-GFP to the apical surface, whereas the manuscript under revision presents only partial abrogation of Centrin-RFP apical docking. Again raising the question, are CLAMP and Centrin migrating BB different in any way?

20. We regret if our 2009 paper was insufficiently clear, but it does not report that Clamp is a basal body-localized protein. Rather, Clamp localizes adjacent to the BB (near the ciliary rootlet) and to the tips of cilia. While the reviewer is correct that Clamp trafficking is disrupted by loss of Fuz, how or even if this relates to localization of Fam92 or other proteins to BBs is unknown.

6. Migrating centrioles are a normal process of MCCs differentiation expected to be found in WT embryos as well? Would these immature cells have less, same or a gradient of IFT-43 GFP as well? Should a comparison be made between immature cells where both WT, mutants and rescues are done in cells with migrating centrioles?

21. This is an important point also made by Reviewer #2 (Points 5 and 6, above). We now show the levels of Ift43, Rsg1, and Fam92 at docked and undocked BB in Fig. 7 and Supp. Fig 7.

7. Mass spec negative control. These results can only display what is different between both pull downs but not what is specific as the negative control is absent. PRIDE links were not shared so data could not be evaluated in review.

22. We regret the oversight! Data are in PRIDE (Project accession: PXD055830).

8. Dominant negative hypothesis. Although slightly tangential to the patient variants being examined, this APMS experiment is super important and completely not

discussed. The RSG1-T69N mutant was generated to create a purported Dominant Negative effect, mentioned again here. If like Ras-S17N (Nassar et al., 2010) RSG1-T69N would sequester upstream activators and would be unable to activate downstream effectors. Regarding this hypothesis (INTU/Fuzz putative RSG1 GEF), then RSG1-T69N would bind INTU/Fuzz, with higher affinity than wild-type RSG1, and would localise to the ciliary base (but this does not seem the case (Agbu et al., 2018)). Moreover the APMS data here fully supports an alternative model.

So it appears RSG1-T69N does not have a dominant negative effect. But RSG1-T69N would be different from the Ras-S17N mutant. RSG1-T69N is probably not in a "GDP-locked" state (Lee et al., 2009; Bos et al., 2007). Asparagine at position 69 would prevent RSG1 binding of Magnesium and would also interfere with GDP/GTP binding. Regarding this hypothesis (INTU/Fuzz putative RSG1 GEF), then RSG1-T69N would be unable to bind INTU/Fuzz putative GEF complex, and then would be unable to localise to the ciliary base (which seems to be the case (Agbu et al., 2018))

23. We regret that the paper was unclear about what was meant by dominant-negative. On the one hand -as we stated in the Introduction of the paper- the cryo-EM structure of CPLANE indicates that Rsg1 is *not* a substrate of the Intu/Fuz GEF but rather binds as an effector. Moreover, the GEFs and GAPs for Rsg1 (if they exist) are unknown. So, when we said Rsg1-T65N has dominant-negative *activity*, we meant *in vivo*. In our 2009 *Nature Cell Biology* paper, we showed that this allele fails to localize to BBs and its expression disrupts ciliogenesis. All that said, to clarify the revision, we have removed the term "dominant negative."

9. Data not shown- either you show the data (Supplemental) or delete all sections with these statements in relation to Fam92A? Does wild type FAM92 localize to TZ BB in *Xenopus* and how is it changed in Rsg1 KDs? Given IFTA is known to scavenge TZ proteins in axoneme- could the phenotype be a secondary?

24. This is another great question, and one raised by Reviewer #2 as well (Point 5, above). We have addressed it in new Fig. 7.

CEP192 is a centrosome marker- how to distinguish from all centrosomes versus those to become basal bodies and fail- say CEP164? Something to mark mother centriole? For example, Fig. 8G- how do you know what a transition zone is in a non-ciliated cell?

25. It is not clear to us exactly what the reviewer is asking. There are a huge number of centrosome markers, and we chose Cep192 because it is reliable. It is not clear to us that testing other centriole markers will provide new information

As for TZ, we identify these using TZ proteins as markers. It is known that the TZ can form even when no cilium forms (e.g. during early ciliogenesis and in *Ift88* mutant cells).

10. Nomenclature throughout. Throughout all the text, figure legends and figures- it is chaos. Human genes upper case italics, human proteins upper case. Mouse genes title

case italics, mouse proteins upper case. *Xenopus* genes appear lower case italics, and proteins title case. It is extremely confusing to readers.

26. We've done our best to correct this!

Minor issues

1. Missing reference on discussion at top of page 7 on hexa-longin GEF complexes:
DOI: 10.1093/bioinformatics/btz739
2. Details of what is being quantified in most graphs? What is each data point- a single centriole (from how many cells, from how many embryos, from how many injection runs) Ideally plotted as super-plots so that means of individual animals and experimental runs can be separated to understand origin of variation. Similarly in the methods, there is a statement which needs clarifying to be informative- 'Each experiment includes multiple biological replicates, conducted on different days.' Biological replicate- different embryos always same injection mix?
3. Plasmids used- no reference to nonGFP-tagged constructs- how to validate expression (localization/function etc)?
4. Lacking data of LAP-tag constructs- what was tagged (human, mouse?), where and single copy/polyclonal etc. Methods say RPE1 and text says IMCD3 cells?
5. Lacking details of MEFs used- primary? Immortalized? From what stage embryo? Reference in Methods too please.
6. Inaccessible proteomics dataset- not publically released and no reviewer key provided. Journal editorial desks should check this ahead of sending out to review to allow datasets to be properly interrogated.

Typos/Edits

1. Tone down the organism 'Top Trumps'- '*Xenopus*, a proven platform for modelling the cell biology of ciliopathies'. Similarly the third paragraph of the introduction, emphasis on species dilutes the understanding of the molecular pathway. OFD is not a conventional motile ciliopathy- why jump to this motile, multiciliated cell type, versus say patient fibroblasts or modelling in human cell line?
2. p.4 3rd paragraph- delete 'that results in a Arg188Trp change in the coding region'- redundant.
3. In the text, the third patient p.ARG188TRP is supposed to relate to Supp Fig 1but this patient in the text is not supposed to have polydactyly? Misreference- Supp Fig Legend refers to patient 2.
4. Typo- delete and 'structure revealed that and A76 anchors the a1'
5. Missing word- if suspected ciliopathy alleles of CPLANE2/RSG1 did likewise.

27. Minor comments corrected.