EMBO reports

Male infertility associated Ccdc108 Regulates Multiciliogenesis via the Intraflagellar Transport Machinery

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DOI: 10.15252/embr.202152775

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Review Timeline:

Submission Date:	2nd Mar 21
Editorial Decision:	22nd Apr 21
Revision Received:	22nd Oct 21
Editorial Decision:	21st Dec 21
Revision Received:	3rd Jan 22
Accepted:	12th Jan 22

Editor: Deniz Senyilmaz Tiebe

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **22nd Apr 2021**

Dear Chris,

Thank you for our recent video chat and submitting your preliminary point-by-point response. I have now looked at your points carefully. I appreciate that you can address many of the concerns raised and see that the proposed experiments will strengthen the manuscript.

Having looked at everything, I would like to invite you to submit a revised manuscript. However, I would like to point out that we need strong support from the referees to consider publication here. It is this aspect that is more difficult to assess at this stage.

Please see the guidelines for the revision below my signature.

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

Kind regards,

Deniz

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

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

As a matter of policy, competing manuscripts published during the revision period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

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Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

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9) Please make sure to include a Data Availability Section before submitting your revision - if it is not applicable, make a statement that no data were deposited in a public database. Primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see).

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

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Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates.
Please also include scale bars in all microscopy images.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

Referee #1:

Review: EMBOR-2021-52775-T
Title: Ccdc108 Regulates Multiciliogenesis via Interaction with the Intraflagellar Transport Machinery

Summary: Solid multipronged work from the Daar and Westlake labs to characterize how Ccdc108, and by association IFT-B, interact to regulate multiciliogenesis. Beautiful advanced imaging, great use of genetic tools and model organisms/systems however the manuscript unfortunately completely fails to engage with all the literature that has come before it. As a result, the authors seem to miss a huge amount/all of the key papers from Chlamy through to human primary ciliary dyskinesia (PCD) genetics which show that CCDC108/CFAP65/DRC2 is a human PCD gene which has a definite structure on EM tomography of motile cilia where it is a hub for assembly of the nexin-dynein regulator complex (N-DRC). This structure is unfortunately completely neglected in the background framing the work, as well as guiding the analysis, interpreting the data and this makes critically reviewing this manuscript very problematic. I believe that this was likely an honest oversight by the authors but one that is a fatal flaw for this manuscript as it currently stands.

A significant amount of the work presented in this manuscript has been done previously with consistently different results; zebrafish depletion (doi:10.1016/j.ajhg.2013.08.015); mammalian multiciliated cells including PCD patients as well as depletion experiments (MMCs- doi:10.1016/j.ajhg.2013.08.015, doi:10.1371/journal.pone.0072299) and Chlamydomonas mutants (doi: 10.1091/mbc.E17-08-0510; DOI: 10.1247/csf.18.371). In all of these studies, loss of CCDC108/CFAP65/DRC2 does not alter the number or length of cilia or flagella but does result in severe defects in cilia beat pattern (dyskinetic or hyperkinetic beat), and very subtle defects in ultrastructure. As such outer dynein arms (ODAs) are not affected, nor are most inner dynein arms (IDAs) except a single subspecies in Chlamy, with a subtle loss of a crescent-shaped- structure over radial spoke 2(RS2)when you look carefully (https://doi.org/10.1016/j.ajhg.2013.08.015) and better resolved by cryoET (doi:10.1371/journal.pone.0072299). In Chlamy, using GFP or HA-tagged DRC2 constructs- there is relatively uniform (albeit possibly regular punctae with a 96nm spacing) staining of the flagellar axonemes (and basal body) with none of the large aggregates reported in this paper that accumulate in the tip (DOI: 10.1247/csf.18.371). This is similar to the endogenous staining reported in mammalian cells (doi:10.1371/journal.pone.0072299). The Chlamy paper from the Porter group importantly also shows that the turnover of DRC2-GFP in a motile flagellum is very slow, much slower than IFT in wild type cells, in keeping with the fact that N-DRC subunits are tightly bound to outer doublet microtubules. There is also work about DRC2 being transported by IFT-B and its trafficking kinetics before it becomes stably integrated into the axoneme which the authors fail to mention (doi: 10.1016/j.cub.2013.10.044), where feedback loops exist with ciliary length/organelle size control. In contrast, the Zhao manuscript using Xenopus morphants and CRISPR knock-outs focuses on decreased ciliation as a result in changes in basal body docking and apical actin reorganization, which is not the phenotypes observed in any of the other model systems nor expected for components which make up structural integrity elements like the N-DRC. As it stands, some of the analysis in the Zhao paper is problematic in its interpretation as the interaction proteomics studies were all performed (there is a lack of specific detail in the methods) by GFP-trap in HEK 293 cells which do not have motile cilia or an N-DRC, nor is the bait normally expressed in non-motile ciliated cells.

However, I recognize that there is an impressive amount of work and quality of the data in this manuscript is significant. As a reviewer, I have spent the last week trying to think of ways help direct the authors with suggestions to bring their analysis in line

with the significant body of work that comes before it. It needs to engage, even if it challenges the previous literature, choosing to be iconoclastic. As such I would recommend the corresponding authors read these references listed above carefully and significantly rejig their introduction and emphasis. There is also a general lack of understanding of the literature cited currently- for example, they cite the Li et al 2020 paper for CFAP65 mutations in male infertility, which also generate a mouse knock-out (which has similar sperm defects), whilst other loss of function mutations lead to PCD (see above), suggesting allelism may be at play for a coiled-coil domain protein. Could this account for the differences the group see here- there are 3 protein coding variants all with UniProt hits- would they all have been targeted with your strategy? Similarly, there is quite a lot of attention focused on DynAPs in this manuscript- however in the work from Wallingford they clearly show that non-dynein components, like DRC2, would likely behave more like radial spoke proteins Rsph1 or Nme1 which localize to the cytoplasm (doi: 10.7554/eLife.38497). Given this, I think it is important that the authors dispel that over-expression on a coiled-coil protein like CCDC108 doesn't result in aggresome formation and hence recruit Reptin and Pontin as protein disaggregases to deal with their disassembly versus being bonafide DynAPs. Your co-localization staining looks like an outer halo of these components as opposed to the dense core staining for DynAps. Similarly in figure 4C- low level punctate of Ccdc108 do not co-localize with Stip1 unless massive accumulations occur, where Stip1 which functions as an adaptor between HSP70 and HSP90 acting as a switch between folding and degradation (https://doi.org/10.1038/s41467-020-19783-w). These cytoplasmic accumulations they report are very large- looking at the size of the IFT-B particles in Figure 5 or the final video- these dwarf the size- they look larger than the 200nm axoneme diameter? To align their work with what came before, cilia beat analysis (not flow) will need to be done- is the beat frequency or waveform altered in the remaining cilia (aligning with previous studies), as well as TEM do the cilia have normal ultrastructure? I would really recommend the authors to reach out to the amazing Mary Porter (University of Minnesota) who is a DRC2 expert and a really helpful member of the cilia community, similarly Win Sale (Emory).

Minor points:
1. Abstract: PCD symptoms sinopulmonary disease versus tracheitis would be more apropos; human PCD with hydrocephaly is quite rare.
2. Abstract: consider '...membrane in epidermal multiciliated cells...'
3. Abstract: change to 'dynein axonemal particles (DynAPs)'... DynAPs should always be plural throughout.
4. Introduction: '...large numbers'... not true nodal motile cilia are singular, the choroid plexus has ~10 motile cilia, whilst ependymal cells have 10s of cilia, the sperm flagellum is also singular.
5. Introduction: '....of most motile cilia has a '9+2'..'- again nodal cilia lack the CP to allow for rotational movement.
6. Introduction: change '...which included axonemal dyneins...' IFT dyneins are also ciliary....
7. Introduction: consider ....'the commitment of MCC precursors'...
8. Introduction: 3 paragraph is problematic: the referencing and narrative is scrambled. A rewrite would likely be helpful. There is quite a lot known about specific IFT-B modules again which haven't been engaged with. Particularly how various IFT-B subunits interact directly with ODA transport proteins including ODA16/DAW1 and IFT46 (N.T. Ahmed, C. Gao, B.F. Lucker, D.G. Cole, D.R. Mitchell ODA16 aids axonemal outer row dynein assembly through an interaction with the intraflagellar transport machinery. J. Cell Biol., 183 (2008), pp. 313-322 Y. Hou, G.B. Witman The N-terminus of IFT46 mediates intraflagellar transport of outer arm dynein and its cargo-adaptor ODA16 Mol. Biol. Cell, 28 (2017), pp. 2420-2433 M. Taschner, A. Mourão, M. Awasthi, J. Basquin, E. Lorentzen Structural basis of outer dynein arm intraflagellar transport by the transport adaptor protein ODA16 and the intraflagellar transport protein IFT46 J. Biol. Chem., 292 (2017), pp. 7462-7473 Mahmoud R. Fassad, Amelia Shoemark, Pierrick le Borgne, France Koll, Mitali Patel, Mellisa Dixon, Jane Hayward, Charlotte Richardson, Emily Frost, Lucy Jenkins, Thomas Cullup, Eddie M.K. Chung, Michel Lemullois, Anne Aubusson-Fleury, Claire Hogg, David R. Mitchell, Anne-Marie Tassin, Hannah M. Mitchison, C11orf70 Mutations Disrupting the Intraflagellar Transport-Dependent Assembly of Multiple Axonemal Dyneins Cause Primary Ciliary Dyskinesia, The American Journal of Human Genetics, Volume 102, Issue 5, 2018, Pages 956-972.
9. Introduction: 'Dynein axonemal assembly factors (DNAAFs)...' these are not inserted into the final dynein subcomplex rather act as chaperones or co-chaperones to help in cytoplasmic pre-assembly process.
10. Introduction: '...axonemal inner and outer dynein arms...' are not referred to as dynein arms until they are docked on the axoneme and visible by TEM- otherwise we just outer arm dyneins or inner arm dyneins.
11. Introduction: Paragraph 5 needs to engage with all of the literature!
12. Nomenclature: human genes are all caps italics, proteins all caps. Mouse genes title case italics, proteins all caps.
13. There is a lot of details missing from the Materials and Methods section: including everything about the mouse ependymal cell culture experiments, the interactomics (datasets should be uploaded onto something like PrideDB with full details of experimental procedures).
14. Given the Chlamy data-it may be worthwhile overexpressing your tagged CCDC108 in knock-out cells (depleted cells) in Xenopus such that every CCDC108 molecule is tagged and where does it now localize? Enriched in cilia, equally along the length? EM gold at the N_DRC throughout? The train phenomenon represents over-expression on top of endogenous levels- sites are saturated?
15. Discussion needs complete overhaul. The final paragraph is wrong.

Referee #2:

Zhao et al. use the Xenopus ciliated epidermis model to elucidate the functions of the ciliopathy gene Ccdc108 in cilia of multi-

ciliated cells. In loss-of-function and rescue studies the authors demonstrate that Ccdc108 is required for normal ciliation and fluid flow production. Ciliogenesis defects originate from abnormal basal body behavior, which fail to dock to the cell membrane, a prerequisite for cilia formation and F-actin organization. They further show that Ccdc108 co-localizes with DynAPs, membrane-less organelles involved in ciliary dynein arm assembly required for ciliary motility. In mass-spec, CoIP and life cell-imaging experiments, they also demonstrate that Ccdc108 interacts with IFT-B proteins that mediate transport of ciliary proteins within the axoneme. These interactions depend on a conserved domain, and constructs harboring mutations in this domain fail to rescue ciliogenesis, basal body docking, and F-actin organization. Lastly, the authors show that loss of IFT74, a Ccdc108 interacting IFT-B protein, causes basal body, F-actin and cilia defects similar to the loss of Ccdc108. From these results, the authors conclude that Ccdc08 and IFT-B complex proteins are required for basal body apical migration and docking, thereby implicating a novel function of IFTs in processes prior to axoneme establishment.
These results constitute novel findings in the field of cilia biology and the presented data is generally of high quality, including beautiful micrographs and solid quantification. Nevertheless, I am not convinced of the author's interpretation that apical transport of basal bodies is really affected. Furthermore, the data as presented in the current form, do not provide mechanistic insight as to why Ccdc108 and IFT-B are required for basal body behavior prior to axoneme extension. Therefore, some additional experiments would be required to further elucidate the underlying molecular mechanism, and the specific questions as well as suggestions for experiments are described below. I think that the group has most tools and methods established, and that they could perform the indicated experiments, which would allow them to resubmit a revised version in a reasonable amount of time for reconsideration.
Major points:
- The authors show effects after Ccdc108/IFT74 knockdown in motile cilia of multi-ciliated cells and immotile sensory cilia of neuromasts, but have not tested if other cilia types are similarly affected. Additional motile monocilia exist in the left-right organizer and primary cilia can be found in the developing neural tube. It would be conceptually important to assess the effects of Ccdc108 and IFT74 loss on these cilia as well. If defects are recovered in all cilia types, these effects could be fully uncoupled from ciliary motility and dynein arm assembly strengthening the author's conclusions.
- It is hard to believe that F-actin network formation is defective, but that the apical membrane size of affected cells remains unchanged. Judging from the reported stage of embryos and their morphology shown in micrographs, the analysis might have been carried out a bit too early. Effects on actin and apical membrane size should be repeated in later stages (stage 32 - 35) when the cells are fully developed. Additionally, it might be interesting to analyze cytoplasmic microtubules (e.g. using GFP-EMBT) to see if those are also affected after knockdowns.
- Connected to the F-actin defects, it remains unresolved why basal bodies fail to organize the F-actin network in morphants. Ciliary adhesions, Cp110, Nphps, Formins and other basal body proteins that were reported to participate in actin network establishment should be analyzed, as they might not localize properly to basal bodies after loss of Ccdc108 and IFT74. A lack of these components would provide an explanation for the observed defects in F-actin establishment.
- The cellular localization of Ccdc108 and IFT74 should be analyzed in different maturation stages of ciliated cells. So far, the authors do not report any localization of these proteins to basal bodies, thus, making it hard to imagine how they could participate in basal body apical transport and docking. The provided images indicate to me that apical transport of basal bodies is not affected, but that apically localized basal bodies fail to dock to ciliary vesicles and the apical membrane. Therefore, showing if and when Ccdc108 localizes to basal bodies could help to identify how it could influence basal body behavior to generate the phenotype. An additional possibility is that basal bodies initially dock but fail to remain docked to the apical membrane. This possibility could also be tested by life-cell imaging of basal bodies in maturing and differentiated ciliated cells.
- In morphants, basal bodies that are able to dock to the membrane seem to form normal cilia. Are these cilia motile and do they contain dynein arms connecting the microtubule doublets? Do these cilia show changes in length?
- From the analysis shown, it remains unclear to me if the mutants of Ccdc108 still localize to DynAPs, basal bodies and/or the transition zone or if they remain simply cytoplasmic. This should be presented more clearly.
Minor points:
- Please provide z-dimension scale bars in lateral projections (e.g. in Fig 3).
- Please provide all sample numbers in the figures or legends (in many cases it is either not stated or just stated that more than X cells were analyzed).
- Please provide some means validating the CRISPR efficiency. At this point, the text simply states that validation was performed but these are not shown.
- Fluorescent intensity quantification was conducted in Photoshop and was normalized based on separately injected control embryos. ImageJ or similar specialized software for image quantification should be used instead, as Photoshop was not made for such purposes. Non-targeted cells from the same embryo should be used for normalization of fluorescent intensities, as this would provide a more stringent reference point than separately injected and stained specimens.
- Overexpression of Ccdc108 seems to induce aggregates in non-ciliated cells. Are these DynAPs, non-specific aggregates or does Ccdc108 localize to specific structures in non-ciliated cells as well?

Referee #3:

The manuscript from Zhao et al addresses the function of Ccdc108 in multiciliogenesis. They provide data primarily from Xenopus but confirmed in zebrafish and mouse that that CCDC108 is important for proper cilia formation. They show that knocking down Ccdc108 (MO or CRISPR) results in a decrease in cilia and furthermore that there is a defect in basal body

apical migration and docking which is combined with a failure to accumulate normal apical actin. They go to show that Ccdc108 localizes to punctate dots in cilia and to DynAPs accumulations in the cytoplasm. They perform Mass spec to identify IFT interacting proteins and confirm this biochemically as well as identifying and deleting the essential IFT interacting domain. They show that expression of mutants that can't bind IFT fail to rescue MO phenotypes and furthermore that loss of IFT74 phenocopies the basal body docking defects. They argue that this implicates IFT in a previously unappreciated role of trafficking basal bodies to the apical surface and that this role requires interactions with Ccdc108. The manuscript is well written and data is in general of high quality and properly controlled. The paper is primarily descriptive and results for the most part represent an incremental advance. While the authors show that Ccdc108 interacts with IFT and that this is important, lots of things interact with IFT and we really don't learn anything about Ccdc108s function. The idea that IFT is important for basal body migration and docking is of potential interest but is very preliminary as presented.

Comments:
1. It is notoriously difficult to quantify cilia number in MCCs. I struggle to believe the quantification of cilia number in Control MO or rescue experiments in 1D. At the very least this needs to be described in much more detail than is currently found in the methods.
2. "Our results revealed that centrioles numbers were similar in control and Ccdc108 morphants (162 {plus minus} 17 in control morphants vs. 153 {plus minus} 17 in Ccdc108 morphants) (Fig 3A)." While I agree that the phenotype is not impressive, the quantified data shows statistical significance. Additionally qualitatively speaking the Cep164 staining does not look as symmetric or robust in the morphants. Therefore I don't think it is appropriate for the authors to claims that Ccdc108 is dispensable for centriole amplification and maturation.
3. In Figure 4A-C the authors argue that the intracellular aggregations of Ccdc108 represent DynAPs, which seems reasonable given the localization with Ruvbl2 and Stip1. However, these images are zoomed in to show only the MCCs. In Figure 6A a broader view shows that all cells have these aggregations. This is a serious issue regarding the interpretation of their data and the importance of these foci. The Huizar et 2018 paper for example shows RuvBl2 specifically enriched in MCCs.
4. Overall the localization of Ccdc108 to cilia is not particularly impressive as presented (although I believe the results). In 4E it is not explained how this imaging was done on motile cilia, without accounting for motility (minor). Given that we can't really see the cilia in this context it is hard to know for certain the veracity of these claims. However, the data from the movie appears much more convincing, so clearly more compelling data exists. A time series where the cilia is more visible should be used.
5. The authors claims that CRISPR of Ift74 was used instead of morpholinos because CRISPR generates mosaic editing. There is a bit of failed logic here. First, MOs can easily be injected mosaicially in a controlled manner. Second the CRISPR data as provided shows pretty universal phenotypes (e.g. not mosaic). Were the quantified images "cherry picked" among regions of the embryos with and without phenotype? If so this should be very carefully explained and a complete data set of all cells should be provided (in EV). If that is not the case then the rationale for using CRISPR should be removed as misleading.
Minor:
1. "We first examined whether Ccdc108 morphants affected centriole amplification and maturation in Xenopus MCCs (Balestra & Gonczy, 2014; Ma et al., 2014; Zhao et al., 2013)." While these references are ok there are much more appropriate references for Xenopus and there are numerous reviews that could be included here.
2. "One possibility is there may be two sub-types or layers of the F-actin network associated with multiciliogenesis." Probably appropriate to reference Werner et al 2011, Antoniades et al. 2014 and Mahuzier et al. 2018 which have argued for two sub-types or layers of actin.

Referee #1

Review: EMBOR-2021-52775-T

Title: Ccdc108 Regulates Multiciliogenesis via Interaction with the Intraflagellar Transport Machinery

Summary: Solid multipronged work from the Daar and Westlake labs to characterize how Ccdc108, and by association IFT-B, interact to regulate multiciliogenesis. Beautiful advanced imaging, great use of genetic tools and model organisms/systems however the manuscript unfortunately completely fails to engage with all the literature that has come before it. As a result, the authors seem to miss a huge amount/all of the key papers from Chlamy through to human primary ciliary dyskinesia (PCD) genetics which show that CCDC108/CFAP65/DRC2 is a human PCD gene which has a definite structure on EM tomography of motile cilia where it is a hub for assembly of the nexin-dynein regulator complex (N-DRC). This structure is unfortunately completely neglected in the background framing the work, as well as guiding the analysis, interpreting the data and this makes critically reviewing this manuscript very problematic. I believe that this was likely an honest oversight by the authors but one that is a fatal flaw for this manuscript as it currently stands.

We thank this reviewer for positive comments about our work. Based on the misunderstanding on the protein being investigated, we have responded to comments specifically related to issues connected to Ccdc108/CFAP65 from the initial comments from this reviewer.

A significant amount of the work presented in this manuscript has been done previously with consistently different results; zebrafish depletion (doi:10.1016/j.ajhg.2013.08.015); mammalian multiciliated cells including PCD patients as well as depletion experiments (MMCs- doi:10.1016/j.ajhg.2013.08.015, doi:10.1371/journal.pone.0072299) and Chlamydomonas mutants (doi: 10.1091/mbc.E17-08-0510; DOI: 10.1247/csf.18.371). In all of these studies, loss of CCDC108/CFAP65/DRC2 does not alter the number or length of cilia or flagella but does result in severe defects in cilia beat pattern (dyskinetic or hyperkinetic beat), and very subtle defects in ultrastructure. As such outer dynein arms (ODAs) are not affected, nor are most inner dynein arms (IDAs) except a single subspecies in Chlamy, with a subtle loss of a crescent-shaped- structure over radial spoke 2(RS2)when you look carefully (https://doi.org/10.1016/j.ajhg.2013.08.015) and better resolved by cryoET (doi:10.1371/journal.pone.0072299). In Chlamy, using GFP or HA-tagged DRC2 constructs- there is relatively uniform (albeit possibly regular punctae with a 96nm spacing) staining of the flagellar axonemes (and basal body) with none of the large aggregates reported in this paper that accumulate in the tip (DOI: 10.1247/csf.18.371). This is similar to the endogenous staining reported in mammalian cells (doi:10.1371/journal.pone.0072299). The Chlamy paper from the Porter group importantly also shows that the turnover of DRC2-GFP in a motile flagellum is very slow, much slower than IFT in wild type cells, in keeping with the fact that N-DRC subunits are tightly bound to outer doublet microtubules. There is also work about DRC2 being transported by IFT-B and its trafficking kinetics before it becomes stably integrated into the axoneme which the authors fail to mention (doi: 10.1016/j.cub.2013.10.044), where feedback loops exist with ciliary length/organelle size control. In contrast, the Zhao manuscript using Xenopus morphants and CRISPR knock-outs focuses on decreased ciliation as a result in changes in basal body docking and apical actin reorganization, which is not the phenotypes observed in any of the other model systems nor expected for components which make up structural integrity elements like the N-DRC.As it stands, some of the analysis in the Zhao paper is problematic in its interpretation as the interaction proteomics studies were all performed (there is a lack of specific detail in the methods) by

GFP-trap in HEK 293 cells which do not have motile cilia or an N-DRC, nor is the bait normally expressed in non-motile ciliated cells.

Most of these comments are related to the misunderstanding of the protein we have studied. As for the concern from the reviewer about our proteomic analysis in HEK293 cells, we believe our results show a specific interaction with IFT-B components as only the wildtype Ccdc108 can bind IFT proteins while mutant Ccdc108 proteins (Δ7 and 7G) fail to do so. We appreciate that 293T cells do not have motile cilia, however, we note this approach enabled the identification of specific interactions with IFT-B proteins that are globally expressed ciliary factors.

However, I recognize that there is an impressive amount of work and quality of the data in this manuscript is significant. As a reviewer, I have spent the last week trying to think of ways help direct the authors with suggestions to bring their analysis in line with the significant body of work that comes before it. It needs to engage, even if it challenges the previous literature, choosing to be iconoclastic. As such I would recommend the corresponding authors read these references listed above carefully and significantly rejig their introduction and emphasis. There is also a general lack of understanding of the literature cited currently- for example, they cite the Li et al 2020 paper for CFAP65 mutations in male infertility, which also generate a mouse knock-out (which has similar sperm defects), whilst other loss of function mutations lead to PCD (see above), suggesting allelism may be at play for a coiled-coil domain protein. Could this account for the differences the group see here- there are 3 protein coding variants all with UniProt hits- would they all have been targeted with your strategy?

We thank the reviewer for catching the missing reference to mouse Ccdc108 KOs in the introduction and we have added references related to the mouse studies, as well as a new paper by Wang et al., (2021). In the discussion, we have also added additional comments related to Ccdc108 knockout mice and potential PCD-links. We did note that the recent Wang et al. paper described developmental delay and small size of mice. These phenotypes could be associated with cilia function, and notably have been described/observed in several PCD mouse models including HYDIN (although not described in the text). However, as we did not perform these studies we feel discussing allelism in this model would be speculative on our part. Our results with shRNA in mouse ependymal cells show that the protein is depleted strongly by several shRNA with similar effect. In the case of *Xenopus*, we designed our morpholinos with the purpose of targeting all known variants of Ccdc108/CFAP65.

Similarly, there is quite a lot of attention focused on DynAPs in this manuscript- however in the work from Wallingford they clearly show that non-dynein components, like DRC2, would likely behave more like radial spoke proteins Rsph1 or Nme1 which localize to the cytoplasm (doi: 10.7554/eLife.38497). Given this, I think it is important that the authors dispel that over-expression on a coiled-coil protein like CCDC108 doesn't result in aggresome formation and hence recruit Reptin and Pontin as protein disaggregases to deal with their disassembly versus being bonafide DynAPs. Your co-localization staining looks like an outer halo of these components as opposed to the dense core staining for DynAps. Similarly in figure 4C- low level punctate of Ccdc108 do not co-localize with Stip1 unless massive accumulations occur, where Stip1 which functions as an adaptor between HSP70 and HSP90 acting as a switch between folding and degradation (https://doi.org/10.1038/s41467-020-19783-w). These cytoplasmic accumulations they report are very large- looking at the size of the IFT-B particles in Figure 5 or the final video- these dwarf the size- they look larger than the 200nm axoneme diameter?

We thank the reviewer for this suggestion related to Ccdc108 localization and DynAPs. We reexamined the localization of Ccdc108 in *Xenopus* embryos that were injected with a lower amount of mRNA and

observed reduced levels of DynAP-like structures (new Fig 4A, 4D). Importantly, centriole, basal body and axoneme localization could now be detected under these conditions. These results suggest that high expression promotes DynAP, or possibly aggresome structures and ciliary puncta localization, which likely obscures detection of Ccdc108 at other cellular structures. Moreover, we found that live cell imaging of GFP-Ccd108 assisted with detecting the protein on centrioles. The localization of Ccdc108 at lower levels of expression observed along the axoneme (new Fig 4A) is consistent with Chlamy-based links of this protein to the axoneme. As the primary focus of the previously submitted manuscript, and the revised manuscript, is on determining how Ccdc108 regulates apical migration, we felt that it would be best to remove the few studies linking Ccdc108 to DynAP (old Fig 4B, 4C and old Ev2A, 2B) as these results are inconclusive, and we have moved the higher expression images of Ccdc108 from the old Fig 4A,D,E to supplementary Fig EV4. Although we cannot rule out Ccdc108-DynAP links, it was not feasible to further examine both Ccdc108-DynAP ciliary relationships and centriole Ccdc108-IFT-B links to actin-dependent centriole migration (new Fig 7 and 8). It would be interesting to investigate potential Ccdc108-DynAP connections in future work, considering the important points about DynAP-radial spoke relationships and the suggested experiments made by this reviewer.

To align their work with what came before, cilia beat analysis (not flow) will need to be done- is the beat frequency or waveform altered in the remaining cilia (aligning with previous studies), as well as TEM do the cilia have normal ultrastructure? I would really recommend the authors to reach out to the amazing Mary Porter (University of Minnesota) who is a DRC2 expert and a really helpful member of the cilia community, similarly Win Sale (Emory).

Given that our results indicate that Ccdc108/CFAP65 functions earlier in ciliogenesis prior to when proteins like Ccdc65/DRC2 function, we predicted it may be challenging to evaluate Ccdc108 function in motility by TEM in the remaining cilia due to complications of partial knockdown effects. We did perform TEM analysis, but we could not make a conclusion about structural alterations of the axoneme/central pair, and therefore we did not include this data. However, to address the comments of Reviewer #1 and #2 on motility, we performed live cell imaging to examine cilia movement in Ccdc108 depleted MCCs and found that both CBP and CBF of the remaining motile cilia were affected by Ccdc108 depletion (Fig EV1D). These results suggest that Ccdc108 is important for ciliary motility, consistent with its proposed function in Chlamy.

Minor points:

1. Abstract: PCD symptoms sinopulmonary disease versus tracheitis would be more apropos; human PCD with hydrocephaly is quite rare.

In the revised manuscript, we have made changes to the text as suggested.

2. Abstract: consider '...membrane in epidermal multiciliated cells...'

We have changed the text following the suggestion.

3. Abstract: change to 'dynein axonemal particles (DynAPs)'... DynAPs should always be plural throughout.

In the revised manuscript, all DynAPs related contents has been removed.

4. Introduction: '...large numbers'... not true nodal motile cilia are singular, the choroid plexus has ~10 motile cilia, whilst ependymal cells have 10s of cilia, the sperm flagellum is also singular.

Thank you for the suggestion, we have made changes to the text in the revised manuscript.

5. Introduction: '....of most motile cilia has a '9+2'..'- again nodal cilia lack the CP to allow for rotational movement.

Thank you for the suggestion, we have made this change to the text.

6. Introduction: change '...which included axonemal dyneins...' IFT dyneins are also ciliary....

Thank you for the suggestion, we have made this change to the text.

7. Introduction: consider ....'the commitment of MCC precursors'...

We have changed the text as suggested.

8. Introduction: 3 paragraph is problematic: the referencing and narrative is scrambled. A rewrite would likely be helpful. There is quite a lot known about specific IFT-B modules again which haven't been engaged with. Particularly how various IFT-B subunits interact directly with ODA transport proteins including ODA16/DAW1 and IFT46 (N.T. Ahmed, C. Gao, B.F. Lucker, D.G. Cole, D.R. Mitchell ODA16 aids axonemal outer row dynein assembly through an interaction with the intraflagellar transport machinery. J. Cell Biol., 183 (2008), pp. 313-322 Y. Hou, G.B. Witman The N-terminus of IFT46 mediates intraflagellar transport of outer arm dynein and its cargo-adaptor ODA16 Mol. Biol. Cell, 28 (2017), pp. 2420-2433 M. Taschner, A. Mourão, M. Awasthi, J. Basquin, E. Lorentzen Structural basis of outer dynein arm intraflagellar transport by the transport adaptor protein ODA16 and the intraflagellar transport protein IFT46 J. Biol. Chem., 292 (2017), pp. 7462-7473 Mahmoud R. Fassad, Amelia Shoemark, Pierrick le Borgne, France Koll, Mitali Patel, Mellisa Dixon, Jane Hayward, Charlotte Richardson, Emily Frost, Lucy Jenkins, Thomas Cullup, Eddie M.K. Chung, Michel Lemullois, Anne Aubusson-Fleury, Claire Hogg, David R. Mitchell, Anne-Marie Tassin, Hannah M. Mitchison, C11orf70 Mutations Disrupting the Intraflagellar Transport-Dependent Assembly of Multiple Axonemal Dyneins Cause Primary Ciliary Dyskinesia, The American Journal of Human Genetics, Volume 102, Issue 5, 2018, Pages 956-972.

We appreciated the comments from the reviewer on the narrative about IFT-B ciliary transport function. However, we felt that because our new results show IFT-B protein localization is regulated by Ccdc108 during the centriole migration of multiciliogenesis that the narrative of this paragraph should be tailored to these findings. Therefore, in the revised manuscript introduction we now describe what is known about IFT recruitment during ciliogenesis rather than on downstream ciliary transport mechanisms.

9. Introduction: 'Dynein axonemal assembly factors (DNAAFs)...' these are not inserted into the final dynein subcomplex rather act as chaperones or co-chaperones to help in cytoplasmic pre-assembly process.

In the revised manuscript, all DynAPs related contents has been removed.

10. Introduction: '...axonemal inner and outer dynein arms...' are not referred to as dynein arms until they

are docked on the axoneme and visible by TEM- otherwise we just outer arm dyneins or inner arm dyneins.

In the revised manuscript, all DynAPs related contents has been removed.

11. Introduction: Paragraph 5 needs to engage with all of the literature!

We believe this suggestion is related to the misunderstanding of the protein we have studied.

12. Nomenclature: human genes are all caps italics, proteins all caps. Mouse genes title case italics, proteins all caps.

In the revised manuscript, we have made changes to the text as suggested.

13. There is a lot of details missing from the Materials and Methods section: including everything about the mouse ependymal cell culture experiments, the interactomics (datasets should be uploaded onto something like PrideDB with full details of experimental procedures).

We apologize for the confusion. Please note that the methodology of mouse ependymal cell culture was included in 'cell culture and transfection' of the method section, and the LC/MS data was included in supplementary Dataset EV1. We have added/expanded experimental procedures where needed in the Method's section.

14. Given the Chlamy data-it may be worthwhile overexpressing your tagged CCDC108 in knock-out cells (depleted cells) in Xenopus such that every CCDC108 molecule is tagged and where does it now localize? Enriched in cilia, equally along the length? EM gold at the N_DRC throughout? The train phenomenon represents over-expression on top of endogenous levels- sites are saturated?

We appreciate the comments by the reviewer on Ccdc108 localization. As noted in an earlier response above, at lower levels of expression we observe Ccdc108 along the axoneme similar to reports in mouse sperm flagella and consistent with what has been proposed in Chlamy. Based on these results and our focus with the revised work examining Ccdc108 function in centriole migration we did not attempt additional immune-EM studies. We agree with the reviewer that the train phenomenon represents over-expression. However, given that the Ccdc108-IFT-B interaction motif is needed for Ccdc108 ciliary localization (Fig 4F, EV3F), the results showing the co-localization and co-transport of Ccdc108-IFTB proteins in IFT-train like structures at high expression suggest IFT may be responsible for transporting Ccdc108 into cilia during axoneme formation.

15. Discussion needs complete overhaul. The final paragraph is wrong.

We appreciate this comment which we believe can be explained by the misunderstanding of the proteins under study in this manuscript. However, we have modified the discussion significantly because of our new findings on Ccdc108 mechanism of function in MCC ciliation.

Referee #1 additional comments:

I apologize for this oversight; please flag this with the authors (this is a consequence of WFH with interruptions).

As they say, the nomenclature is very confusing. What kicked off my rabbit hole, was that this is AKAP240 is a component of the central pair complex in Chlamy and appears to be the mammalian equivalent as well. See attached and find CFAP65 here: http://chlamyfp.org/readcsvfile_js_Pfam.php. (Gaillard AR, Diener DR, Rosenbaum JL, Sale WS. Flagellar radial spoke protein 3 is an A-kinase anchoring protein (AKAP). J Cell Biol. 2001;153(2):443–8.) In mutants lacking the central pair, there is no AKAP240 and in this paper, they find it localized to C2 microtubule of the central apparatus (https://bmcmolcellbiol.biomedcentral.com/articles/10.1186/s12860-016-0103-y). This complex is confusing and somewhat obtuse- not well understood- but has many studies from the Sale and D'Souza lab in Chlamydomonas this needs anchoring to.
It has been mapped more recently to central pair protein proteomes and substructures: doi: 10.2142/biophysico.BSJ-2019048 and doi: 10.1083/jcb.201902017 I think the issue is central pair proteins are not thought to affect cilia formation, rather function, although moonlighting roles are possible.

I own my mistakes and frazzled brain. I did, to my embarrassment segue, into similar sounding but unrelated proteins that PCD genes and components of the N-DRC which again would not be thought to affect cilia formation, rather function. But the point still stands, this work needs to be anchored with the Chlamy and other unicellular organisms which is extensive- George Witman, Khanh Bui, Jacinta DeSouza would all be well placed to advise.

Usually CP mutants like HYDIN, doi: 10.1016/j.ajhg.2012.08.016, which localizes to lack the C2b projection of the central pair (CP) apparatus cause PCD and sperm abnormalities, no defects in cilia number. This is what is confusing here.

There is a huge amount of work in the central pair apparatus and what this does should be brought forward- you mention the Rao paper but not how it affects cilia motility. It is in a sentence with DynAPs in the discussion.

We appreciate the comments on the Gaillard paper and other CFAP65/Ccdc108 central pair associated publications. We have added additional references and comments to the manuscript to better anchor our work to previous studies in Chlamy and CFSP65/Ccdc108 central pair function. In the revised manuscript, as mentioned in response to the previous comments/suggestion from the reviewer, we provide several new studies that demonstrate a ciliogenesis function for Ccdc108 upstream of the axoneme in organizing centriolar proteins needed for the actin-dependent migration of centrioles to the cell surface. Moonlighting roles for Ccdc108 upstream of axonemal assembly are also supported by a study by Wang et al, (2021) that was published after our initial submission to this journal, which showed requirements of Ccdc108 in sperm acrosome assembly, which we have cited.

Again these types of proteins would not be expected to be going through DynAPs but how they are assembled and turned over remains unclear.

As noted we have removed DynAP from the manuscript.

I am just trying to help this paper align itself with iconoclastic findings. It is beautiful work, trying to maximize impact.

We greatly thank the Reviewer for all their efforts and helpful comments which we believe will enhance the impact of this work.

Referee #2

Zhao et al. use the Xenopus ciliated epidermis model to elucidate the functions of the ciliopathy gene Ccdc108 in cilia of multi-ciliated cells. In loss-of-function and rescue studies the authors demonstrate that Ccdc108 is required for normal ciliation and fluid flow production. Ciliogenesis defects originate from abnormal basal body behavior, which fail to dock to the cell membrane, a prerequisite for cilia formation and F-actin organization. They further show that Ccdc108 co-localizes with DynAPs, membrane-less organelles involved in ciliary dynein arm assembly required for ciliary motility. In mass-spec, CoIP and life cell-imaging experiments, they also demonstrate that Ccdc108 interacts with IFT-B proteins that mediate transport of ciliary proteins within the axoneme. These interactions depend on a conserved domain, and constructs harboring mutations in this domain fail to rescue ciliogenesis, basal body docking, and F-actin organization. Lastly, the authors show that loss of IFT74, a Ccdc108 interacting IFT-B protein, causes basal body, F-actin and cilia defects similar to the loss of Ccdc108. From these results, the authors conclude that Ccdc08 and IFT-B complex proteins are required for basal body apical migration and docking, thereby implicating a novel function of IFTs in processes prior to axoneme establishment.

These results constitute novel findings in the field of cilia biology and the presented data is generally of high quality, including beautiful micrographs and solid quantification. Nevertheless, I am not convinced of the author's interpretation that apical transport of basal bodies is really affected. Furthermore, the data as presented in the current form, do not provide mechanistic insight as to why Ccdc108 and IFT-B are required for basal body behavior prior to axoneme extension. Therefore, some additional experiments would be required to further elucidate the underlying molecular mechanism, and the specific questions as well as suggestions for experiments are described below. I think that the group has most tools and methods established, and that they could perform the indicated experiments, which would allow them to resubmit a revised version in a reasonable amount of time for reconsideration.

We thank the reviewer for their positive comments about our manuscript and for helpful suggestions aimed at gaining mechanistic insight into CCDC108/IFT-B function in multiciliogenesis.

Major points:

- The authors show effects after Ccdc108/IFT74 knockdown in motile cilia of multi-ciliated cells and immotile sensory cilia of neuromasts, but have not tested if other cilia types are similarly affected. Additional motile monocilia exist in the left-right organizer and primary cilia can be found in the developing neural tube. It would be conceptually important to assess the effects of Ccdc108 and IFT74 loss on these cilia as well. If defects are recovered in all cilia types, these effects could be fully uncoupled from ciliary motility and dynein arm assembly strengthening the author's conclusions.

We thank the reviewer for this suggestion. We examined Ccdc108 functioning in non-motile ciliogenesis in *Xenopus* embryos and find that Ccdc108 is dispensable for the formation of primary cilia in the neural tube and mono motile cilia in the gastrocoel roof plate (GRP) (new Fig EV1E, F). Our results support a role for Ccdc108 in MCCs that can be uncoupled from its downstream cilia motility function (new EV1D). Given Ccdc108 is not important for ciliation in these cells we did not investigate IFT74 function in these cell types.

- It is hard to believe that F-actin network formation is defective, but that the apical membrane size of affected cells remains unchanged. Judging from the reported stage of embryos and their morphology shown in micrographs, the analysis might have been carried out a bit too early. Effects on actin and apical

membrane size should be repeated in later stages (stage 32 - 35) when the cells are fully developed. Additionally, it might be interesting to analyze cytoplasmic microtubules (e.g. using GFP-EMBT) to see if those are also affected after knockdowns.

We thank the reviewer for this insightful comment. Thus, we performed this analysis at stage 32 as suggested and found that Ccdc108 depleted MCCs of embryos at this stage displayed a significantly reduced apical size (Fig 3C vs. Fig EV2B). This result is consistent with Ccdc108 morphant failure to maintain the expanded apical surface due to the reduction of the F-actin network and ciliation and supports our previous assertion that Ccdc108 functions in F-actin network regulation.

We examined GFP-EMBT in MCCs and did not observe noticeable effects. But we did not carry out quantitative analysis of these results to be included in the manuscript because of the striking effects observed with the actin cytoskeleton at stage 28 and 32 and additional new studies described at stage 18 in Fig 8, which were are carried out related to the next suggestion below from the reviewer.

- Connected to the F-actin defects, it remains unresolved why basal bodies fail to organize the F-actin network in morphants. Ciliary adhesions, Cp110, Nphps, Formins and other basal body proteins that were reported to participate in actin network establishment should be analyzed, as they might not localize properly to basal bodies after loss of Ccdc108 and IFT74. A lack of these components would provide an explanation for the observed defects in F-actin establishment.

We thank the reviewer for these suggestions. We examined the effects of Ccdc108 depletion on ciliary adhesion proteins Fak and Cp110, and actin cytoskeletal regulators Drg1 and the RBD, an indicator of activated RhoA, in *Xenopus* embryos at stage 18 (a stage used resulting from the reviewers next suggestion below). Ccdc108 depletion significantly reduced the centriolar accumulations of Drg1 and RBD but not the ciliary adhesion proteins (Fig 8A-D). We did not believe we could evaluate IFT74 CRISPR effects on these processes due to expected issues with determining areas of protein depletion resulting from mosaic embryos, which we could more easily identify at later developmental stages using cilia or centriole markers as shown in Fig 6. However, because we can show that Ccdc108-IFT interaction is required for IFT-B protein accumulation at the centrioles during migration (Fig 7C-E) at stage 18, we could indirectly test this question using Ccdc108 IFT binding motif mutants that lack the domain necessary for an interaction with IFT. These Ccdc108 mutants were used in rescue studies examining Drg1 and RBD centriolar localization. Strikingly, the Ccdc108 IFT-B mutants failed to promote accumulation of actin cytoskeletal regulators (Drg1, active RhoA) to migrating centrioles (Fig8C,8D). Together these studies support a mechanism whereby Ccdc108 cooperates with IFT-B proteins to regulate actin centriolar cytoskeleton regulators needed for proper centriole migration during multiciliogenesis.

- The cellular localization of Ccdc108 and IFT74 should be analyzed in different maturation stages of ciliated cells. So far, the authors do not report any localization of these proteins to basal bodies, thus, making it hard to imagine how they could participate in basal body apical transport and docking. The provided images indicate to me that apical transport of basal bodies is not affected, but that apically localized basal bodies fail to dock to ciliary vesicles and the apical membrane. Therefore, showing if and when Ccdc108 localizes to basal bodies could help to identify how it could influence basal body behavior to generate the phenotype. An additional possibility is that basal bodies initially dock but fail to remain docked to the apical membrane. This possibility could also be tested by life-cell imaging of basal bodies in maturing and differentiated ciliated cells.

We once again appreciate the insightful suggestions made by the reviewer aimed at understanding Ccdc108 molecular mechanism in centriole migration. In the revised manuscript, we report new discoveries into Ccdc18 localization resulting from lower expression of the proteins than used in the previously submitted manuscript – this point is further discussed in the response to the DynAP comment made by this reviewer below and to Reviewer #1 and #3. Examination of the subcellular localization of Ccdc108 and IFT-B proteins in MCCs of embryos at non-ciliated stage 18 and ciliated stage 27 (Kulkarni *et al*, 2021) demonstrated Ccdc108 proteins can localize to migrating centrioles and the basal body (Fig 4A, D, 7A). Importantly, IFT-B proteins show a similar localization (Fig 4D, 7B). Ccdc108 depletion significantly reduces the centriolar accumulation of IFT-B proteins at stage 18 (Fig 7C-E) and centriolar accumulation of actin cytoskeletal regulators (Fig 8C, 8D), while Ccdc108 IFT binding motif mutants fail to restore the defects (Fig 7C-E, 8C, 8D). Together results indicate that Ccdc108 depletion affects ciliogenesis during a stage when centrioles are migrating. We appreciate the suggestion about undocking issues, but we feel our studies demonstrate the primary defect in ciliogenesis occurs before the docked stage. However, we also recognize that we cannot rule out that centriole retention at the apical surface membrane is impaired. Evaluating this phenomenon is likely to be challenging given the observed centriolar migration issues, and therefore is a question that we feel would be better addressed in a separate study specifically examining centriole retention at the cell surface.

- In morphants, basal bodies that are able to dock to the membrane seem to form normal cilia. Are these cilia motile and do they contain dynein arms connecting the microtubule doublets? Do these cilia show changes in length?

We appreciate this comment. To address this question raised by Reviewers #1 and #2, we performed live cell imaging to examine cilia motility in Ccdc108 depleted MCCs and found that both CBP and CBF of the remaining motile cilia were indeed impaired by Ccdc108 depletion (Fig EV1D). We also confirmed that Ccdc108 depletion resulted in slightly shorter cilia in MCCs using scanning electron microscopy (SEM) (EV1B). As cilia can still beat, we presume at least a partially functional dynein arm structure is present. As noted in our response to Reviewer #1, our TEM analysis was not conclusive about structural alterations and therefore we did not include this data.

- From the analysis shown, it remains unclear to me if the mutants of Ccdc108 still localize to DynAPs, basal bodies and/or the transition zone or if they remain simply cytoplasmic. This should be presented more clearly.

We appreciate the suggestion from the reviewer. As mentioned in a previous response to the comment from this reviewer, and in more detail in our response to Reviewer #1, when we examined the localization of Ccdc108 in embryos by injecting with a lower amount of mRNA we showed that Ccdc108 localizes along the ciliary axoneme and basal body in ciliated MCCs (Fig 4A). Based on our new analysis we observe wildtype Ccdc108 and IFT-interaction mutants at migrating centrioles and basal bodies with live cell imaging. Examination of transition zone localization would have required super resolution imaging which was not possible as fixed cells displayed a weak GFP-Ccd108 signal (not shown). We also believe at higher levels of mRNA the high signal observed for Ccdc108 concentrated in cytoplasmic structures and the ciliary puncta prevented observation of the protein elsewhere in MCCs. Based on these new observations we have removed DynAPs related references from the manuscript as we cannot rule out this was an effect of aggregation resulting from overexpression. It is possible that Cdc108 is associated with DynAPs but additional studies that would require a significant time to confirm this result more definitively. During the revision period, we focused on investigating Ccdc108-IFT-B mechanisms in centriole migration regulation.

Minor points:

- Please provide z-dimension scale bars in lateral projections (e.g. in Fig 3).

Thank you for the suggestion. We have made changes to the z-dimension scale bar.

- Please provide all sample numbers in the figures or legends (in many cases it is either not stated or just stated that more than X cells were analyzed).

Thank you for the suggestion. We have provided a source data file containing all the statistic information.

- Please provide some means validating the CRISPR efficiency. At this point, the text simply states that validation was performed but these are not shown.

Following this suggestion, a supplementary figure (Fig EV5) has been included to show an example of the in vitro validation of the CRISPR/Cas9 mediated genome editing.

- Fluorescent intensity quantification was conducted in Photoshop and was normalized based on separately injected control embryos. ImageJ or similar specialized software for image quantification should be used instead, as Photoshop was not made for such purposes.

We understand the concern of the reviewer, and we compared analysis in Photoshop to Image J for a few image series and got very similar results. Based on these results we hope the reviewer understands it would have been a significant amount of work to repeat all these analyses again. Additionally, please see the comment below about how normalizations were performed.

Non-targeted cells from the same embryo should be used for normalization of fluorescent intensities, as this would provide a more stringent reference point than separately injected and stained specimens.

We appreciate the reviewer' suggestion. However, we did not use the uninjected cells from the same embryo to normalize. We followed the protocol published to do normalization, which is used by others in *Xenopus* field (Arnold *et al*, 2019) and we have now cited this paper.

- Overexpression of Ccdc108 seems to induce aggregates in non-ciliated cells. Are these DynAPs, non-specific aggregates or does Ccdc108 localize to specific structures in non-ciliated cells as well?

We agree with the reviewer that these structures are sometime observed in non-ciliated cells which would not be expected for DynAPs, a point also made by Reviewer #3. For this reason, and the points outlined in above response to the reviewer and to Reviewer #1 and #3, we feel significant effort outside of the scope of the revised work would be needed to conclusively demonstrate Ccdc108-DynAP links, which was not intended to be the primary focus of the previously submitted manuscript. For the revised manuscript, we have instead focused on mechanistic studies evaluating Ccdc108 role with the IFT-B complex in centriole migration. As noted previously, we have removed all DynAP-related data (old Fig 4b, 4C and old Ev2A, 2B) and text descriptions.

Referee #3

The manuscript from Zhao et al addresses the function of Ccdc108 in multiciliogenesis. They provide data primarily from Xenopus but confirmed in zebrafish and mouse that that CCDC108 is important for proper cilia formation. They show that knocking down Ccdc108 (MO or CRISPR) results in a decrease in cilia and furthermore that there is a defect in basal body apical migration and docking which is combined with a failure to accumulate normal apical actin. They go to show that Ccdc108 localizes to punctate dots in cilia and to DynAPs accumulations in the cytoplasm. They perform Mass spec to identify IFT interacting proteins and confirm this biochemically as well as identifying and deleting the essential IFT interacting domain. They show that expression of mutants that can't bind IFT fail to rescue MO phenotypes and furthermore that loss of IFT74 phenocopies the basal body docking defects. They argue that this implicates IFT in a previously unappreciated role of trafficking basal bodies to the apical surface and that this role requires interactions with Ccdc108. The manuscript is well written and data is in general of high quality and properly controlled. The paper is primarily descriptive and results for the most part represent an incremental advance.

We want to thank the reviewer for their comments regarding the quality and rigor of our work. We apologize if we did not clearly state the important advances of our work in our initial submission which includes, the first investigation of the function of disease associated Cfap65/Ccdc108 protein in ciliogenesis (at the time of submission), the first study to implicate Ccdc108 and the IFT-B complex in MCC centriole migration, and identification of a specific IFT-B complex interaction motif on Ccdc108 needed for centriole migration and Ccdc108 ciliary localization. In our revised manuscript, we have added new experiments (new Fig 4A, 4D, Fig 7 and 8) that confirm the requirement for a Ccdc108-IFT interaction in centriole migration via affecting recruitment of actin cytoskeleton regulators to these migrating centrioles. Importantly, our work shows that IFT-B complex protein recruitment to centrioles requires Ccdc108 via its identified IFT-B binding motif; to our knowledge this is the first demonstration of a specific mechanism for how IFT-B proteins are recruited to centrioles in MCCs, or for that matter any ciliated cells, during ciliogenesis.

While the authors show that Ccdc108 interacts with IFT and that this is important, lots of things interact with IFT and we really don't learn anything about Ccdc108s function. The idea that IFT is important for basal body migration and docking is of potential interest but is very preliminary as presented.

We appreciate the comment from the reviewer about the importance of the IFT connection to Ccdc108. While we agree IFT interacts with many different proteins we believe the identification of the Ccdc108-IFT-B interaction is of unique significance. As noted in the first response to the comment from this reviewer, in the revised version of the manuscript, we provide new detailed functional insight into Ccdc108 function as a factor needed to recruit IFT-B proteins to the centrioles at a stage when they are migrating in MCCs (new Fig 7). Moreover, we demonstrate that Ccdc108 and its interaction with IFT-B complex are needed to recruit the PCP associated actin cytoskeleton factors Drg1 and the activated RhoA to the centrioles (Fig 8), but are dispensable for ciliary adhesion complex proteins Cp110 and Fak centriole accumulation. This provides a plausible explanation for why the apical actin cytoskeleton is affected in later stage MCCs and for why centriole docking to the apical membrane is dysregulated.

Comments:

1. It is notoriously difficult to quantify cilia number in MCCs. I struggle to believe the quantification of cilia number in Control MO or rescue experiments in 1D. At the very least this needs to be described in much more detail than is currently found in the methods.

We apologize for not explaining how this analysis was performed. Using SIM super resolution microscopy, we have found it is possible to identify individual cilium by observing the axoneme staining (Ac-tub) and distal appendage staining (Cep164) at the cilia base. Related information has been provided in the method section of the revised manuscript.

2. "Our results revealed that centrioles numbers were similar in control and Ccdc108 morphants (162 {plus minus} 17 in control morphants vs. 153 {plus minus} 17 in Ccdc108 morphants) (Fig 3A)." While I agree that the phenotype is not impressive, the quantified data shows statistical significance. Additionally qualitatively speaking the Cep164 staining does not look as symmetric or robust in the morphants. Therefore I don't think it is appropriate for the authors to claims that Ccdc108 is dispensable for centriole amplification and maturation.

We agree a slight difference in centriole numbers exists. However, when considering the strong effect on ciliation we observe this small change in centriole number is unlikely to explain the ciliogenesis dysfunction observed. We have rephrased the text to help clarify this point in the revised manuscript.

With respect to centriole maturation as gauged by CEP164, because the centrioles are not uniformly docked to the apical membrane the random orientation of the centrioles in the cytoplasm affects the structures observed by super resolution SIM. However, CEP164 signal is observed in proximity of the majority of cytoplasmic centrioles (Fig 1D, 1F, 3A and 2H) which suggests that distal appendages are able to form. We have added an inset image for the CEP164 channel to Fig 3A Ccdc108MO treatments to better show the Cep164 relationship with the Centriole marker (Cetn1).

3. In Figure 4A-C the authors argue that the intracellular aggregations of Ccdc108 represent DynAPs, which seems reasonable given the localization with Ruvbl2 and Stip1. However, these images are zoomed in to show only the MCCs. In Figure 6A a broader view shows that all cells have these aggregations. This is a serious issue regarding the interpretation of their data and the importance of these foci. The Huizar et 2018 paper for example shows RuvBl2 specifically enriched in MCCs.

We agree with the reviewer that Ccdc108 foci can sometimes be observed in neighboring cells, although this is less prominently than in MCCs. As noted in the responses to the other reviewers, we reexamined the localization of Ccdc108 in *Xenopus* embryos that were injected with a lower amount of mRNA and observed reduced levels of DynAP-like structures, and interestingly we could observe the protein at centrioles, basal bodies and along the axoneme in live cell imaging studies. Because our main objective was to understand Ccdc108 requirements in centriole migration we decided to focus our attention during the revision period on the more direct links to this process with Ccdc108-IFT-B. While we cannot rule out a link between Ccdc108-DynAP in MCC ciliogenesis, it was just not feasible to investigate both Ccdc108-IFT-B and Ccdc108-DynAP during this revision period. Consequently, we have decided to remove DynAP related data (old Fig 4b, 4C and old Ev2A, 2B) given the questions/concerns of the reviewers.

4. Overall the localization of Ccdc108 to cilia is not particularly impressive as presented (although I believe the results). In 4E it is not explained how this imaging was done on motile cilia, without accounting for motility (minor). Given that we can't really see the cilia in this context it is hard to know for certain the veracity of these claims. However, the data from the movie appears much more convincing, so clearly more compelling data exists. A time series where the cilia is more visible should be used.

We apologize for not describing how Ccdc108 cilia transport was performed in the old Fig 4E (now EV3C). Live embryos were mounted in agarose, and therefore less mobile, and were imaged by spinning disk confocal microscopy. As noted in the response to the Reviewer #1's Point #3, Ccdc108 puncta in cilia is associated with higher levels of expression, and at lower expression levels it localizes along the ciliary axoneme (new Fig 4A) consistent with a recent report in mouse sperm flagella (Wang et al., 2021), and its reported association with axoneme central pair in Chlamy.

5. The authors claims that CRISPR of Ift74 was used instead of morpholinos because CRISPR generates mosaic editing. There is a bit of failed logic here. First, MOs can easily be injected mosaic ally in a controlled manner. Second the CRISPR data as provided shows pretty universal phenotypes (e.g. not mosaic). Were the quantified images "cherry picked" among regions of the embryos with and without phenotype? If so this should be very carefully explained and a complete data set of all cells should be provided (in EV). If that is not the case then the rationale for using CRISPR should be removed as misleading.

We appreciate the reviewers comment about knockdown strategies for Ift74. We attempted Ift74 knockdown in *Xenopus* MCCs using two different MOs and observed strong lethality that could not be rescued suggesting off-target effects. We have noted this in the results section of the revised manuscript. The reviewer makes an excellent point regarding CRISPR mosaicism issues and approaches that can be used. We apologize for this oversight in describing our approach and we now included a statement in the method section and added Figure EV5 to better illustrate our approach used. As noted in the revised manuscript, due to known mosaic effects when CRISPR/Cas9 system is applied to embryos (Mehravar *et al*, 2019), fields displaying phenotypic defects in the crispant samples were imaged identified by effects on cilia, or centriole markers, while similar defects were rarely observed in a field of cells in control samples. These areas of affected MCCs were usually larger than the microscopy imaging field, which typically contained 8-10 MCCs.

Minor:

1. "We first examined whether Ccdc108 morphants affected centriole amplification and maturation in Xenopus MCCs (Balestra & Gonczy, 2014; Ma et al., 2014; Zhao et al., 2013)." While these references are ok there are much more appropriate references for Xenopus and there are numerous reviews that could be included here.

We thank the reviewer for these suggestions. In the revised manuscript, additional references have been incorporated into the text as suggested.

2. "One possibility is there may be two sub-types or layers of the F-action network associated with multiciliogenesis." Probably appropriate to reference Werner et al 2011, Antoniades et al. 2014 and Mahuzier et al. 2018 which have argued for two sub-types or layers of actin.

We thank the reviewer for these suggestions. In the revised manuscript, these references have been incorporated into the text.

1st Revision - Editorial Decision **21st Dec 2021**

Dear Ira,
Dear Chris,

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

I apologize for the delay in getting back to you. It took longer than anticipated to receive the referee reports.

As you can see, the referees find that the study is significantly improved during revision and recommends publication. However, I need you to address the editorial points below before I can accept the manuscript.

• Please address the remaining minor concerns of the referees.
• We note that the text in the Data Availability section is not accurate. As per our guidelines, the Data Availability section is reserved for the new primary dataset that is generated in this study and deposited in a public data repository. If this is not applicable, please make a statement that no data were deposited in a public database, and the remove the current text.
• Please rename the "Declaration of Interests" as "Conflict of Interests".
• Please make sure that the funding information is complete in both the manuscript text and the manuscript submission system.
• We note that the panels of Fig EV5 are not called out in the text.
• The movies need to be ZIPed with their legends. The movie legends should be removed from the Manuscript file.
• The source data need to be splitted to 1 file per figure. Multiple file types for one figure can be ZIPed.
• Papers published in EMBO Reports include a 'synopsis' and 'bullet points' to further enhance discoverability. Both are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb and 3-5 bullet points listing the key experimental findings.
• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz

--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

Review: EMBOR-2021-52775-R1
Title: Ccdc108 Regulates Multiciliogenesis via Interaction with the Intraflagellar Transport Machinery

Summary: The revised manuscript from the Daar and Westlake labs has substantial new data to back-up new mechanism, and most importantly much more streamlined narrative which better aligns itself with what has come before and what is uncovered in the current study. Multisystem, multipronged approach to get at novel functions specifically for CCDC108/CFAP65 in multiciliated cells beyond its currently described role in the central pair apparatus C2a projection which appears conserved to all motile ciliated types. I congratulate the authors on their efforts in this revised manuscript which is now very compelling- I have some textual edits, particularly to the new text that would help readers better follow the data and frame the novelty to increase impact. Authors should be able to turn this around in a few days.

Text edits:
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p6: Second paragraph first sentence: Consider adding something like 'Together with recent tomography and proteomic studies suggesting CCDC108 is a component of the C2a central apparatus projection, these genetic studies suggest that CCDC108...

However, what its cellular functions are remain unclear. To address this, we investigated requirements and dynamics of Ccdc108 during vertebrate ciliogenesis.' Emphasize the novelty of this study much more explicitly. I would also cite Hou et al 2021 paper from the Witman lab PMID: 33988244, which elevates your story much more from a 'same gene, new organism' type report.

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P9: last line: Consider 'without affecting levels of expression of other ciliary proteins'. Again explicit to emphasize loss of localization of IFT-Bs rather than altering levels.

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P50: Figure EV1 legend 'depletion on motile monocilia formation'

P52: Figure EV4 legend: (B) Capitalized P for one of the p values- rest are all lower case.

Referee #2:

In the revised manuscript, the authors have responded adequately to the criticism and suggestions made by the reviewers. Particularly, they have addressed my main request and significantly expanded on the mechanistic insights. They have also added controls and improved on some technical aspects, which raised the quality of the already beautiful dataset. They now provide extensive evidence that Ccdc108 and IFT-B complex proteins localize to newly produced centrioles, basal bodies and cilia in multiciliated cells of the Xenopus epidermis. They show that Ccdc108 recruits IFT-B components (especially IFT74) and F-actin promoting polarity associated factors, which collectively allows for apical transport, basal body docking, cilia formation, and apical/subapical F-actin network formation in ciliated cells. They further demonstrate that in contrast to multiciliated cells, Xenopus mono-cilia do not require Ccdc108. Collectively this study provides important and novel insights into cilia formation and ciliopathy mechanisms relevant to the cell, cilia and developmental biology communities as well as to clinicians. The study should be accepted after a few minor edits were made.
1. In the abstract, they write: "Moreover, Ccdc108 is required for the centriolar recruitment of Drg1 and activated RhoA, factors that help establish the apical actin network that is essential for centriole migration in MCCs." I think that statement is not fully correct. Drg1 and RhoA most likely help to regulate behavior of F-actin fibers along which centrioles migrate apically. Only after apical localization of basal bodies, proteins associated with them can engage in organizing the apical and subapical F-actin networks. So, I would argue that apical centriole migration is required for apical F-actin network formation and not the other way round.

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When introducing deuterosome function in centriole amplification, they have omitted to cite DevCell Klos Dehring et al. 2013, a hallmark study in Xenopus multiciliated cells.
3. The authors sometimes use the term "multicilia" when referring to cilia of multiciliated cells. Since multicilia is a genus of amoebozoa, I think it would be better to simply say cilia instead. In all cases it is clear from the context that they refer to cilia of multiciliated cells.
4. While the authors now demonstrate that Ccdc108 LOF causes apical surface reduction in mature cells at stage 32, they still suggest that IFT74 LOF does not lead to smaller apical surfaces although F-actin formation is equally affected. If they have only analyzed that at the earlier time point, they should state that specifically or simply remove the claim from page 15 (Together, these results suggest that the Ift74 functions in multiciliogenesis independently of apical surface expansion of MCCs.).

5. Discussion page 22-23: "Furthermore, the function of Ccdc108 in regulating centriolar accumulations of IFT-B proteins and cytoskeleton regulators associated with the PCP pathway might be specific to multiciliogenesis since either primary cilia or monocilia formation was affected by Ccdc108 depletion in Xenopus embryos."
The authors do show that loss of Ccdc108 in zebrafish affects lateral line kinocilia, which are monocilia. Therefore, I would add that information specifically or remove the statement.
"This is supported by our observation that IFT-B proteins are observed on centrioles during the migration stage when Cp110 is present, while in primary ciliogenesis IFT-B protein recruitment to the mother centriole coincides with CP110 uncapping and axoneme growth (Goetz et al., 2012; Kanie et al., 2017; Lu et al., 2015)."
I think the statement is problematic for the following reason: Cp110 pools at ciliary adhesions regulating basal body interactions with F-actin are not localized to the distal end cap (Elife Walentek et al. 2016). The same paper showed that Cep97 does not localized to basal bodies in MCCs. So, I think it would be worth mentioning to avoid making an overstatement on the differences in IFT recruitment and Cp110 removal time-point from the distal end. If I recall correcty (but I am not 100% sure), the Meunier lab has data indicating that Cp110 dissociates from MCC centiole distal ends around the time of deuterosome disengagement (Nature Meunier et al. 2014). So that would be also before apical BB transport as observed by the authors. In any case, I think that the above interpretation is questionable without further evidence.
6. Did I missed it or are there no supplemental videos of the ciliary beating analysis? If those are really missing, please add those to the supplemental section.
7. The image analysis using Photoshop is still problematic to me. The use should be clearly stated in the figure legends, and comparison to ImageJ analysis (as indicated in the response to reviewers) should be presented in the supplementary material section.

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We sincerely appreciate the recognition of our work by the reviewer. Their thorough and constructive suggestions are very helpful to improve our story.

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We thank the reviewer for this suggestion. We have changed this sentence to the following: Given the similarity between IFTs and Ccdc108 in ciliary-associated localization, we investigated whether overexpressed Xenopus Ccdc108 may interact with IFTs using the non-motile ciliated mammalian HEK293T cell line.

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We thank the reviewer's recognition of our manuscript.

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multicilia is a genus of amoebozoa, I think it would be better to simply say cilia instead. In all cases it is clear from the context that they refer to cilia of multiciliated cells.

We thank the reviewer for the suggestion and have made changes to the text.

4. While the authors now demonstrate that Ccdc108 LOF causes apical surface reduction in mature cells at stage 32, they still suggest that IFT74 LOF does not lead to smaller apical surfaces although F-actin formation is equally affected. If they have only analyzed that at the earlier time point, they should state that specifically or simply remove the claim from page 15 (Together, these results suggest that the Ift74 functions in multiciliogenesis independently of apical surface expansion of MCCs.).

We thank the reviewer for the suggestion and have removed the claim from the text.

5. Discussion page 22-23: "Furthermore, the function of Ccdc108 in regulating centriolar accumulations of IFT-B proteins and cytoskeleton regulators associated with the PCP pathway might be specific to multiciliogenesis since either primary cilia or monocilia formation was affected by Ccdc108 depletion in Xenopus embryos."
The authors do show that loss of Ccdc108 in zebrafish affects lateral line kinocilia, which are monocilia. Therefore, I would add that information specifically or remove the statement.

We thank the reviewer for the suggestion and have made changes to the text as suggested by 1# and 2# referees.

"This is supported by our observation that IFT-B proteins are observed on centrioles during the migration stage when Cp110 is present, while in primary ciliogenesis IFT-B protein recruitment to the mother centriole coincides with CP110 uncapping and axoneme growth (Goetz et al., 2012; Kanie et al., 2017; Lu et al., 2015)."
I think the statement is problematic for the following reason: Cp110 pools at ciliary adhesions regulating basal body interactions with F-actin are not localized to the distal end cap (Elife Walentek et al. 2016). The same paper showed that Cep97 does not localized to basal bodies in MCCs. So, I think it would be worth mentioning to avoid making an overstatement on the differences in IFT recruitment and Cp110 removal time-point from the distal end. If I recall correcty (but I am not 100% sure), the Meunier lab has data indicating that Cp110 dissociates from MCC centiole distal ends around the time of deuterosome disengagement (Nature Meunier et al. 2014). So that would be also before apical BB transport as observed by the authors. In any case, I think that the above interpretation is questionable without further evidence.

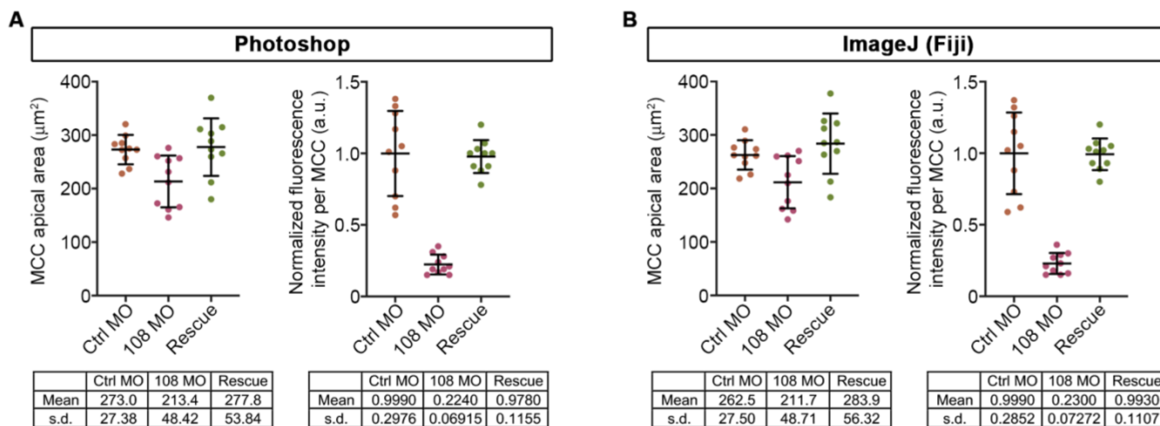
We appreciate the reviewers points. Since we did not analyze the precise localization of CP110 and CEP97 at centrioles or examine whether/when CP110 is removed from the centrioles during multiciliogenesis, we decided to remove this sentence.

6. Did I missed it or are there no supplemental videos of the ciliary beating analysis? If those are really missing, please add those to the supplemental section.

We apologize for not including the related videos and have added them to the supplemental section.

7. The image analysis using Photoshop is still problematic to me. The use should be clearly stated in the figure legends, and comparison to ImageJ analysis (as indicated in the response to reviewers) should be presented in the supplementary material section.

As we have indicated in the methods section that Photoshop was used for our analysis we hope the reviewer will understand not restating this in the legends. Although ImageJ is widely used for image analysis, Photoshop is also commonly used for quantitative measurement of fluorescence intensity (Arias-Hervert et al., 2020; Ghosh et al., 2019; Kirkeby and Thomsen, 2005; Puerta-Guardo et al., 2020). Thus based on published examples we do not believe inclusion of a comparison of ImageJ and Photoshop analysis in the supplementary section is warranted. However, to demonstrate the similar determinations for these two programs for the reviewer, we have included an example of phalloidin fluorescence intensity of individual MCC in our response (below). We analyzed ten images of each sample which shows the same trend.



A – Photoshop, MCC apical area (μm²):

	Ctrl MO	108 MO	Rescue
Mean	273.0	213.4	277.8
s.d.	27.38	48.42	53.84

A – Photoshop, Normalized fluorescence intensity per MCC (a.u.):

	Ctrl MO	108 MO	Rescue
Mean	0.9990	0.2240	0.9780
s.d.	0.2976	0.06915	0.1155

B – ImageJ (Fiji), MCC apical area (μm²):

	Ctrl MO	108 MO	Rescue
Mean	262.5	211.7	283.9
s.d.	27.50	48.71	56.32

B – ImageJ (Fiji), Normalized fluorescence intensity per MCC (a.u.):

	Ctrl MO	108 MO	Rescue
Mean	0.9990	0.2300	0.9930
s.d.	0.2852	0.07272	0.1107

Reference

1. Arias-Hervert, E.R., Xu, N., Njus, M., Murphy, G.G., Hou, Y., Williams, J.A., Lentz, S.I., Ernst, S.A., and Stuenkel, E.L. (2020). Actions of Rab27B-GTPase on mammalian central excitatory synaptic transmission. Physiol Rep *8*, e14428. 10.14814/phy2.14428.
2. Ghosh, A., Bhattacharjee, S., Chowdhuri, S.P., Mallick, A., Rehman, I., Basu, S., and Das, B.B. (2019). SCAN1-TDP1 trapping on mitochondrial DNA promotes mitochondrial dysfunction and mitophagy. Sci Adv *5*, eaax9778. 10.1126/sciadv.aax9778.
3. Kirkeby, S., and Thomsen, C.E. (2005). Quantitative immunohistochemistry of fluorescence labelled probes using low-cost software. J Immunol Methods *301*, 102-113. 10.1016/j.jim.2005.04.006.
4. Puerta-Guardo, H., Tabata, T., Petitt, M., Dimitrova, M., Glasner, D.R., Pereira, L., and Harris, E. (2020). Zika Virus Nonstructural Protein 1 Disrupts Glycosaminoglycans and Causes Permeability in Developing Human Placentas. J Infect Dis *221*, 313-324. 10.1093/infdis/jiz331.

Dear Chris,
Dear Ira,

Thank you for submitting your revised manuscript. I have now looked at everything and all is fine. Therefore, I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice work!

Kind regards,

Deniz
--
Deniz Senyilmaz Tiebe, PhD
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EMBO Reports

--

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Quantification results from at least three independent experiments were subjected to unpaired two-tailed t-test to determine statistical significance.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	All experiments were repeated in at least six embryos, and multiple cells from each embryo were analyzed in the study.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	In samples of CRISPR/Cas9 mediated gene editing embryos only fields displaying phenotypic defects were imaged, while similar defects were rarely observed in control samples. It's well known that CRISPR/Cas9 system can have mosaic effects when applied to embryos (Mehravar et al, 2019). A statement was provided in the method seciton. For the other experiments, no exclusion was performed. Tables of individual numerical values were provided in the Source Data file.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	All samples were treated equally except the CRISPR/Cas9 samples.
For animal studies, include a statement about randomization even if no randomization was used.	Yes. A statement was provided in the method section.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Yes. A statement was provided in the method section.
5. For every figure, are statistical tests justified as appropriate?	Yes. Related information of the unpaired t-test and p-values are included in figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The statistical methods we used are described in the method section and figure legends. Analysis were performed using the Prism 8 software (GraphPad Software). P < 0.05 was considered significant.

Is there an estimate of variation within each group of data?	Quantification results are presented as mean ± s.d unless specified in the figure legend.
Is the variance similar between the groups that are being statistically compared?	For the unpaired t-test, assum both populations have the same SD.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Information of commercial antibodies are included in the method section. The specificity of the home-made antibody was validated by shRNA mediated knockdown of the target protein.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293T cell line was routinely tested for myocoplasma contamination.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Information of related animals are provided in the method section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Mouse, Xenopus and zebrafish experimental procedures were performed in accordance with the protocols (ASP #17-041, ASP #20-433 and ASP#20-416) approved by the animal care & use committee of the National Cancer Institute at Frederick in compliance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes we followed the ARRIVE Guidelines.

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	A Data Availability section and a soure data file were provided.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Raw data of mass spectrometry analysis were provided in Dataset EV1 and the source data file.
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G- Dual use research of concern

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