Interactions between TULP3 tubby domain and ARL13B amphipathic helix promote lipidated protein transport to cilia

Venkata Palicharla, Sun-hee Hwang, Bandarigoda Somatilaka, Issei Shimada, Emilie Legué, Nicole Familiari, Vanna Tran, Jeffrey Woodruff, Karel Liem, and Saikat Mukhopadhyay

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RE: Manuscript #E22-10-0473
TITLE: Interactions between TULP3 tubby domain and ARL13B amphipathic helix promote lipidated protein transport to cilia

Dear Mukhopadhyay,

As you can see from the reviews, both reviewers were positive about this work but each made substantial comments to improve the manuscript. I will be happy to re-evaluate a revised manuscript. Note that reviewer two submitted their comments as a pdf. If the comments in the text below are not clear, please request a copy of the pdf from the MBoC Editorial office.

Sincerely,

Gregory Pazour
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Mukhopadhyay,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you haveopted out of publishing the review history.

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

This is a thorough and well-executed study which goes into great detail about the direct interaction between TULP3 and

ARL13B. The authors report the novel finding that an amphipathic helix at the N terminus of ARL13B is responsible for directly interacting with the TULP3 tubby domain. Through this interaction, ARL13B is brought to the cilia where it can perform its normal functions, including the regulation of other lipidated protein localization to the cilia. Mostly, the data are of high quality and support the conclusions made by the authors. My major criticism of the manuscript is that it is written in a choppy and hard to follow manner. I found it challenging to read. One example is on line 87 where the sentence, "ARL13B is a cytosolic protein that is palmitoylated (Cevik et al., 2010)", comes completely out of the blue.

Other comments:
1. In Figure 1, the immunofluorescence images that you show of ARL13b being mislocalized from the cilia in Tulp3 KO MEFs and 3T3 cells leaves the reader thinking that the protein is not even expressed because there is essentially zero background signal. It would be great to show western blots of ARL13B levels, as you do in Figure 2 for IMCD3 cells, for these two cell lines as well.
2. Line 155 - To substantiate this claim and strengthen the paper you should add tubby and/or other tubby protein family members to the direct interaction experiments in Figure 6.
3. Line 167 - The image shown in Figure S2C shows a complete lack of a SNAP signal after 5 hours in Tulp3 KO cells. What could you be measuring? ARL13b isn't in the cilia at the start.
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6. Figure 5D,E - Why do you only precipitate a small fraction of the total ARL13B? Is there a large molar excess of ARL13B over your GST construct? Is it a weak interaction? A positive control would be nice to show (e.g. Arl3-T31N).
7. Figure 6B - All three blots should be vertically aligned as they are in Figure 6D.
8. Figure S5C - Do you have a negative control?

Reviewer #2 (Remarks to the Author):

This is a well written paper with clear addition to our collective knowledge about lipidated cargo transport to cilia. I appreciate this manuscript's polish and completeness. I'm confident with a few adjustments & additions, this work will be suitable and of great interest to MBoC readers. All comments offered herein are in good spirit to both help the authors and future readers reproduce these experiments.

Summary
This manuscript teases apart the mechanisms of ARL13B transport to cilia via TULP3. Palicharla et al present strong and convincing data that the N-terminal amphipathic helix of ARL13B interacts with TULP3 tubby domain. This interaction requires TULP3 binding to IFT-A but not to phoshoinositides, as is required with TULP3-mediated transport of integral proteins. This work also shows the ARL13B-TULP3 interaction is required for the ciliary localization of downstream, lipidated & ARL3-dependent cargoes of ARL13B (e.g., INPP5E) in both cell lines and mouse.

Major points
Scoring of ciliary protein localization: Much of the data in the paper is based on qualitative assessment of cilia + for protein of interest with no mention of how this scoring and analysis was completed. Only the quantitative analysis was mentioned in M & M. Please include a section describing how this analysis was completed. Were these blinded experiments (as would be desired with subjective analysis)? If in fact these experiments were quantitative, please include threshold for deeming cilia pos or neg. With so much of this paper based on this approach, we really need to have clear details in the M & M.
CRISPR/Cas9 KOs: Please flesh out "Generation of Tulp3 knock out cell lines" (Ln 505). Are you using RNP, lentiviral, plasmid, or other? Transfection method? PCR primer seq for region flanking guide site? Please indicate how/if the genotypes were determined from such messy Sanger traces (Fig S1). Based on these chromatograms, how are you confident you've generated a KO with biallelic disruptive events? This manuscript needs some show evidence KOs were achieved. This reviewer would like to see control chromatograms here too & immunoblots as indicated in the manuscript Ln 511.
Minor points
Ln 71: Obesity is not a common JS phenotype. The Thomas et al 2015 paper cited describes one person with well-defined JS who was obese & acknowledges it's rarely seen in JS. Please omit.
Ln 89: ARl13B ARL13B
Ln 241: CLSs' CLSs
Ln 259: Please define MBP
Ln 285: mutant with "an" alanine substitution

Ln 297 ARL1BV359A ARL13BV358A
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Figure 1: Panel E (& Fig 2 Panel C) is this the acetyl α-tub ab or just α-tub? If acetyl, please indicate appropriately. If alpha, please add to "Antibodies & reagents" in M & M
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Figure 3: If available, would like to see other panels, esp since INPP5E is higher P0, then drops dramatically
Figure 3: Panel D, scale bar is 5 μm?
Figure 4: Panel A: consider putting red x over IFT or PIP2 binding motif for ease of interpretation
Figure 4, Panel B: Please define asterisk in AcTub channel for first imaging series
Figure 5: Please add densitometry to M & M. It's unclear how you're showing stats on densitometry with n=2. Consider including blot replicates used for presented stats in the supplement (here & other applicable figures).
Ln 992: Pam Palm as shown in figure
Figure 7: define other asterisks
Ln 1023: Provide rationale that N=mutations in your chromatograms or adjust wording. Presumably via NHE-J you've made some mutation that caused a frameshift, but likely not induced dozens of mutations, yes?
Figure S2: AcTUB staining here is not great. How are you sure these round puncta are in fact cilia?
Figure S3: define stats
Figure S3: Panel A Arl13bHnn/Hnn Arl13bhnn/hnn
Ln 1067: "ns, not significant" not relevant to this figure
Consistency issues:
N/C-term or N/C term
Coiled coiled vs coiled coil (should be this, yes?)
Notes: This reviewer would much prefer to see actual data points instead of obscured data with bar graphs for future publications.
MBoC requirement: source of all cell lines, method of cell line authentication, & frequency of testing for mycoplasma contamination-please add to M & M

We wholeheartedly thank the reviewers for their comments and constructive critique. We appreciate the very positive response from you and all reviewers (for e. g., Reviewer 1: “thorough and well-executed study”, “data are of high quality and support the conclusions made by the authors”; Reviewer 2: “well written paper with clear addition to our collective knowledge about lipidated cargo transport to cilia”, “great interest to MBoC readers”).

We believe that we have now addressed most of the remaining concerns, and extensively revised the manuscript according to the suggestions. These changes have resulted in the following changes:

- Addition of two supplemental Figs S2 and S5.
    - Fig. S2 shows bi-allelic insertion-deletion mutations in *Tulp3* ko CRISPR lines and lack of disruption of ARL13B cellular content in *Tulp3* ko CRISPR lines and MEFs.
    - Fig. S5 shows images of ARL13B and INPP5E localization in P0 and P24 stages of *Tulp3* conditional ko kidney epithelia.
- Revision of Suppl Figs S3, S4, S6 and S7.
    - Fig. S3C shows revised images of SNAP-ARL13B ciliary localization experiments.
    - Fig. S4D shows additional rescue data in *Tulp3* ko CRISPR lines.
    - Fig. S6A shows *in vitro* binding between Tubby and ARL13B like TULP3.
    - Fig. S7C shows negative control (GST beads).

Revised text in the manuscript is shown in yellow.

Reviewer #1 (Remarks to the Author): This is a thorough and well-executed study which goes into great detail about the direct interaction between TULP3 and ARL13B. The authors report the novel finding that an amphipathic helix at the N terminus of ARL13B is responsible for directly interacting with the TULP3 tubby domain. Through this interaction, ARL13B is brought to the cilia where it can perform its normal functions, including the regulation of other lipidated protein localization to the cilia. Mostly, the data are of high quality and support the conclusions made by the authors. My major criticism of the manuscript is that it is written in a choppy and hard to follow manner. I found it challenging to read. One example is on line87 where the sentence, "ARL13B is a cytosolic protein that is palmitoylated (Cevik et al., 2010)", comes completely out of the blue.

Other comments:

1. In Figure 1, the immunofluorescence images that you show of ARL13b being mislocalized from the cilia in Tulp3 KO MEFs and 3T3 cells leaves the reader thinking that the protein is not even expressed because there is essentially zero background signal. It would be great to show western blots of ARL13B levels, as you do in Figure 2 for IMCD3 cells, for these two cell lines as well.

We have now included western blots of ARL13B levels for all *Tulp3* ko CRISPR lines, and *Tulp3* ko MEFs in Fig. S2B. These data, in addition to the previous data on IMCD3 *Tulp3* ko in Fig 2C, clearly show that total cellular levels of ARL13B protein are unaffected in *Tulp3* ko cells.

2. Line 155 - To substantiate this claim and strengthen the paper you should add tubby and/or other tubby protein family members to the direct interaction experiments in Figure 6.

We tested interactions between ^{GST}Tubby and in-vitro translated ^{Myc}ARL13B and find binding comparable to ^{GST}TULP3. These data are included in Fig. S6A.

3. Line 167 - The image shown in Figure S2C shows a complete lack of a SNAP signal after 5 hours in Tulp3 KO cells. What could you be measuring? ARL13b isn't in the cilia at the start.

We are not exactly sure what the reviewer is referring to. The intensity measurements show no significant differences between all the time points in *Tulp3* ko and the minimal SNAP signal quantified after 5 h in *Tulp3* ko cells possibly reflects background fluorescence.

4. Line 179-180 - In the figure legend, specify that you are only measuring the cilia that have detectable expression.

Thanks for the correction. We mentioned in the legend that we measured fluorescence in cilia having detectable localization.

5. Line 245 - What does the localization of these constructs look like in the cell? Likely, the constructs that go to the cilia, can also biotinylate Tulp3. For these experiments to be meaningful, a control would be needed that goes to the cilia but does not biotinylate Tulp3. I think these BirA experiments (Figure 5A-C) should be moved to the supplement. Proximity labeling may be useful for showing that two proteins do not interact but has little value towards showing that two proteins have a direct interaction.

The proximity biotinylation experiments provide a readout of proximity between TULP3 and cargoes in an *ex vivo* context, so we are inclined to keep these data in the main text. We have previously shown controls for proximity between TULP3 and Fibrocystin and GPCR ciliary localization sequence (CLS) mutants (Badgandi *et al.*, 2017). In the current manuscript, we show some of these controls (CD8 linker, no biotin treatment etc.) and additional controls, such as no proximity between ARl13B and TULP3 N-terminal fragment.

We also have determined *TULP3* tubby domain mutants (such as *K389I)* that prevent proximity with cargoes, such as ARL13B and Fibrocystin CLS. These TULP3 mutants do not affect 4,5 phosphoinositide or IFT-A binding. Describing these *TULP3* tubby domain mutants are beyond the scope of the current paper, but we show a representative result below for TULP3^{K389I} for the reviewer, further arguing for specificity of these proximity biotinylation assays.

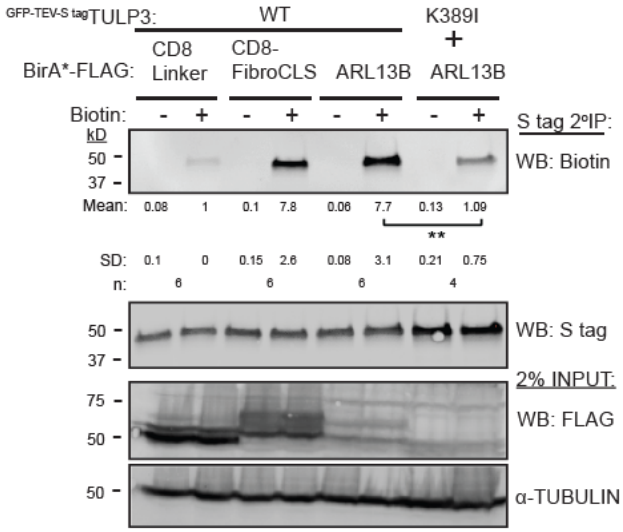

Fig. R1. Lysine K389 in TULP3 tubby domain is involved in interaction with its cargoes. T-Rex 293 cells were co-transfected with GFP-TEV-S-tagged TULP3 wild type (WT) or K389I

mutant along with BirA*-Flag tagged ARL13B and processed as in Figure 5A. Mean ± SD values indicate Biotin/S-tag ratios normalized to CD8 linker control.

6. Figure 5D,E - Why do you only precipitate a small fraction of the total ARL13B? Is there a large molar excess of ARL13B over your GST construct? Is it a weak interaction? A positive control would be nice to show (e.g. Arl3-T31N).

We calculated the % Myc-ARL13B IPs to be up to 20% of input for GST tagged TULP3, within the expected range for *in vitro* binding reactions for binding to TULP3 for other proteins (Badgandi *et al.*, 2017). Regarding the positive control suggested by the reviewer, a recent preprint suggests that GST-ARL3 T31N is not efficient in binding to mammalian ARL13B from cell extracts (Travis *et al.*, 2022). Rather, to fulfill the conditions for binding between ARL13B and ARL3 in our *in vitro* assays using mammalian ARL13B, we would have to test (a) GTP/non hydrolyzable GTP analogue-bound ARL3, (b) other fast cycling variants such ARL3 Y90C (Travis *et al.*, 2022), and (c) in presence of co-GEFs such as BART (ElMaghloob *et al.*, 2021). We respectfully suggest that these experiments are beyond the scope of the current paper and would not provide additional insight into binding between ARL13B and TULP3.

7. Figure 6B - All three blots should be vertically aligned as they are in Figure 6D.

Thanks for the suggestion. We have aligned the blots according to the reviewers' suggestions.

8. Figure S5C - Do you have a negative control?

We have shown the negative control (GST beads only) for this particular experiment in Fig. S7C (previous Fig. S5C). We have also shown extensive negative controls (GST beads only, TULP3-N terminus) and competition with ^{MBP}TULP3 and ^{MBP}Tubby in the *in vitro* binding assays in Fig. 5D-E and revised Fig S6B. Results are shown quantified in multiple assays in all these experiments including those in Fig. S7C (previous Fig. S5C).

Reviewer #2 (Remarks to the Author): This is a well written paper with clear addition to our collective knowledge about lipidated cargo transport to cilia. I appreciate this manuscript's polish and completeness. I'm confident with a few adjustments & additions, this work will be suitable and of great interest to MBoC readers. All comments offered herein are in good spirit to both help the authors and future readers reproduce these experiments.

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desired with subjective analysis)? If in fact these experiments were quantitative, please include threshold for deeming cilia pos or neg. With so much of this paper based on this approach, we really need to have clear details in the M & M.

We have added a Methods section on quantifying ciliary localization of cargoes in Pg 23. In most cases, we also provide quantitative intensity measurements as in Figures 2, 4.

CRISPR/Cas9 KOs: Please flesh out "Generation of Tulp3 knock out cell lines" (Ln 505). Are you using RNP, lentiviral, plasmid, or other? Transfection method? PCR primer seq for region flanking guide site? Please indicate how/if the genotypes were determined from such messy Sanger traces (Fig S1). Based on these chromatograms, how are you confident you've generated a KO with biallelic disruptive events? This manuscript needs some show evidence KOs were achieved. This reviewer would like to see control chromatograms here too & immunoblots as indicated in the manuscript Ln 511.

We have now included TULP3 western blot for all CRISPR lines in Fig. S2. We also rescued IMCD3 *Tulp3* ko CRISPR line with $^{Myc/GFP}$TULP3 as shown in Figs 2 and 4, ruling out non-specific defects. We now added data showing rescue in a second *Tulp3* ko line in IMCD3 cells in Fig S4D. As suggested by the reviewer, we have also updated the Methods section with relevant details for generating the CRISPR ko lines. We used a pLenti-CRISPR Puromycin construct and used limiting dilution to derive single ko clones by western blotting for TULP3 levels. Initial sequencing in controls and ko lines was performed on PCRs using primers flanking Exon 3 of *Tulp3*. We performed TOPO cloning to identify the sequences of the biallelic disruptions and this data has been included in Fig. S2. Control sequencing has now also been shown.

Minor points
Ln 71: Obesity is not a common JS phenotype. The Thomas et al 2015 paper cited describes one person with well-defined JS who was obese & acknowledges it's rarely seen in JS. Please omit.

Thanks, corrected in line 70.

Ln 89: ARl13B to ARL13B

Thanks, corrected.

Ln 241: CLSs' to CLSs

Thanks, corrected.

Ln 259: Please define MBP

Thanks, defined as maltose binding protein.

Ln 285: mutant with "an" alanine substitution

Thanks, but we prefer as we had it before as we are qualifying multiple hydrophobic/charged residues.

Ln 297 ARL1BV359A to ARL13BV358A

Thanks, we retained the previous version as we are mentioning human ARL13B^{V359A} 's interaction with TULP3. Please note that the RVxP motif (sequence R^{357}VEPV^{361}) in mouse ARL13B is identical to R^{358}VEPL^{362} of human ARL13B.

Ln 316: sentence is a bit hard to read, consider "The D211A mutant fragment, with alanine substitution of charged and hydrophobic residues in the amphipathic helix but predicted to retain palmitoylation motif, had reduced binding to TULP3."

Thanks, corrected.

Ln 360: TULP3 to *TULP3*

Thanks, corrected.

Ln 362: insert "and" before Cys1

Thanks, corrected.

Ln 374: interactions "are" mediated by an Ln 439-440 "both make & female mice were analyzed in all experiments" doesn't mesh with Fig 1Panel G where 1 control & 2 cko mice were evaluated & please confirm In Fig 3 sexes were equally balanced

In Figure 3 in the embryonic-onset *Tulp3* models all pups were evaluated irrespective of gender. Sexual dimorphism is not seen in embryonic onset cystogenesis. For e.g., early-onset PKD after conditional *Pkd1* inactivation (Bukanov *et al.*, 2012) or in other PKD mouse models (Natoli *et al.*, 2010) is sex-independent. We have revised this sentence to say that “animals were analyzed irrespective of sex” (Line 442).

Ln 949 "2-4 mice"

We kept it as before, not sure what we need to revise here.

Ln 470-1: Were PIP strips used??

No, we apologize for the oversight. We removed PIP strips from Methods.

Ln 519: human TULP3 and GAPDH to murine Tulp3 and Gapdh, yes?

Thanks, corrected to *Tulp3* and *Gapdh.*

Ln 579: missing space between g & for

Thanks, corrected.

Ln 593: "6000 RPM" Please change to g for ease of reproducibility

Corrected to 5000 *g*.

Ln 618: With multiple hypothesis testing, you should then report adjusted p-values, yes? Please adjust figure legends accordingly

Thanks for the correction. We added adjusted p-values in the legends to Figs 1J and 7C.

Ln 620: Please include a M & M section about WBs

We added the section on immunoblotting in Methods. Immunoblotting while performing proximity biotinylation was previously described in the section on biotinylation.

Figure 1: Panel C It's a bit surprising an underpowered non-parametric test would yield significant difference between these two very similar groups of data points, but perhaps the differences are obscured by the graphical representation. Please confirm Mann Whitney-U gives sig results here.

We confirmed that the shown statistical analyses are accurate.

Figure 1: Panel E (& Fig 2 Panel C) is this the acetyl α-tub ab or just α-tub? If acetyl, please indicate appropriately. If alpha, please add to "Antibodies & reagents" in M & M

We used an hFAB Rhodamine Anti-Tubulin (Bio-Rad; 12004166) antibody for imaging using a BioRad Chemidoc MP imaging system. We changed the labeling to tubulin.

Figure 1: Panel J: Schematic indicates ~27-29 hrs post confluency & legend says 36 hours-please correct whichever

We apologize for the oversight. We corrected the legend to timings mentioned in the schematic.

Figure 1: Panel J: Please indicate how many times this experiment was conducted

We added in the legends that the data is representative of 2 experiments.

Ln 923: LAPINPP5E to GFPINPPE?

Thanks for the suggestion. We changed LAP to GFP for ease of interpreting data in the legends.

Ln 933: >30 cilia to n>30 cilia for consistency

Thanks, corrected.

Figure 2 legend: Ln 924 & elsewhere: confluence to confluent

We retained “confluence” as we are talking about cells being grown till confluence.

Figure 2: Panel A: consider including definition of red AAs for naïve readers in legend

Thanks, we have now included a line in the figure legend (line 938) stating that the red aa signify critical residues in the stated motifs.

Figure 2: Panel C, Bands here for ARL13B & α-TUB look very similar & are at the same kD so presumably on different membranes-please double check to ensure a panel wasn't aberrantly duplicated

The bands shown are accurate. We use the IR-dye mediated detection for blotting against two proteins and simultaneously use hFAB Rhodamine Anti-Tubulin to detect tubulin in the same blot using a BioRad Chemidoc MP imaging system. Adjacent bands thus might look similar.

Figure 3: If available, would like to see other panels, esp since INPP5E is higher P0, then drops dramatically

We have now added images from additional time panels for ARL13B, INPP5E ciliary localization in Fig S5. P0 and P24 time points are shown (P5 time points for ARL13B and INPP5E ciliary localization are shown in Fig. 3).

Figure 3: Panel D, scale bar is 5 μm?

We apologize for the error. We updated the scale bar in the left panels of Fig 3D.

Figure 4: Panel A: consider putting red x over IFT or PIP2 binding motif for ease of interpretation

Thanks for the suggestion, but we prefer the present configuration of the cartoon.

Figure 4, Panel B: Please define asterisk in AcTub channel for first imaging series

Asterisk refers to a cytokinetic bridge. We now mentioned it in legends.

Figure 5: Please add densitometry to M & M. It's unclear how you're showing stats on densitometry with n=2. Consider including blot replicates used for presented stats in the supplement (here & other applicable figures).

We have included densitometry to the Methods section describing immunoblotting in lines 597-8. We showed data as mean+/- SD. It is not possible to show experimental data from up to 6 experiments, thus we quantified the data in respective panels. We have shown replicates for similar datasets in multiple panels for both IVT binding (e.g., Fig 5E, 6B, 6D and S6B) and proximity biotinylation (e.g., Fig. 5B, 5C, S7B) in main and supplemental figures.

Ln 992: Pam to Palm as shown in figure

Thanks, we changed to “Palm” in legends.

Figure 7: define other asterisks

We apologize for the omission. We have now defined the other asterisks.

Ln 1023: Provide rationale that N=mutations in your chromatograms or adjust wording. Presumably via NHE-J you've made some mutation that caused a frameshift, but likely not induced dozens of mutations, yes?

We have now shown allelic mutations in Fig S2 as detailed in page 4 in response to major comment 2.

Figure S2: AcTUB staining here is not great. How are you sure these round puncta are in fact cilia?

We have now improved these images in revised Fig. S3.

Figure S3: define stats

We apologize for the omission. We have now defined the asterisks (now in Fig S4).

Figure S3: Panel A Arl13bHnn/Hnn to Arl13bhnn/hnn

Thanks for the correction. We corrected the labels (now in Fig S4).

Ln 1067: "ns, not significant" not relevant to this figure Consistency issues: N/C-term or N/C term Coiled coiled vs coiled coil (should be this, yes?)

Thanks, we corrected to “coiled coil” in two places.

Notes: This reviewer would much prefer to see actual data points instead of obscured data with bar graphs for future publications.

We have now showed data points for all bar graphs. We already showed data points for cilia lengths and violin plots for fluorescence intensities.

MBoC requirement: source of all cell lines, method of cell line authentication, & frequency of testing for mycoplasma contamination-please add to M & M

We already mentioned sources of cell lines. We have now added method for mycoplasma testing in the cell lines section. We also added a key resource table for reagents in supplemental material.

References

Badgandi, H.B., Hwang, S.H., Shimada, I.S., Loriot, E., and Mukhopadhyay, S. (2017). Tubby family proteins are adapters for ciliary trafficking of integral membrane proteins. J Cell Biol *216*, 743-760.

Bukanov, N.O., Moreno, S.E., Natoli, T.A., Rogers, K.A., Smith, L.A., Ledbetter, S.R., Oumata, N., Galons, H., Meijer, L., and Ibraghimov-Beskrovnaya, O. (2012). CDK inhibitors R-roscovitine and S-CR8 effectively block renal and hepatic cystogenesis in an orthologous model of ADPKD. Cell Cycle *11*, 4040-4046.

ElMaghloob, Y., Sot, B., McIlwraith, M.J., Garcia, E., Yelland, T., and Ismail, S. (2021). ARL3 activation requires the co-GEF BART and effector-mediated turnover. Elife *10*.

Natoli, T.A., Smith, L.A., Rogers, K.A., Wang, B., Komarnitsky, S., Budman, Y., Belenky, A., Bukanov, N.O., Dackowski, W.R., Husson, H., Russo, R.J., Shayman, J.A., Ledbetter, S.R., Leonard, J.P., and Ibraghimov-Beskrovnaya, O. (2010). Inhibition of glucosylceramide accumulation results in effective blockade of polycystic kidney disease in mouse models. Nat Med *16*, 788-792.

Travis, A.M., Manocha, S., Willer, J.R., Wessler, T.S., Skiba, N.P., and Pearring, J.N. (2022). Disrupting the ciliary gradient of active Arl3 affects rod photoreceptor nuclear migration. bioRxiv, 2022.2005.2002.490367.

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