The ARF GAPs ELMOD1 and ELMOD3 act at the Golgi and Cilia to Regulate Ciliogenesis and Ciliary Protein Traffic

Rachel Turn, Yihan Hu, Skylar Dewees, Narra Devi, Michael East, Katherine Hardin, Tala Khatib, Joshua Linnert, Uwe Wolfrum, Michael Lim, James Casanova, Tamara Caspary, and Richard Kahn

Corresponding author(s): Richard Kahn, Emory University

Review Timeline:

| Event                | Date       |
|----------------------|------------|
| Submission Date      | 2021-09-16 |
| Editorial Decision   | 2021-10-18 |
| Revision Received    | 2021-11-16 |
| Accepted             | 2021-11-16 |

Editor-in-Chief: Matthew Welch

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. The original formatting of letters and referee reports may not be reflected in this compilation.)
Dear Dr. Kahn:

Two experts in the field evaluated your manuscript. They found the data over all convincing and the outcome very interesting. Both reviewers have also raised some concerns relating to the quantification of the data, missing information about sample sizes, statistical analyses etc. and the lengthy introduction and discussion.

I'm convinced you can easily address all reviewers' concerns and I'm looking forward to receiving a revised version of your manuscript.

Sincerely,
Anne Spang
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Kahn,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) 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 have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,
Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org
Reviewer #1 (Remarks to the Author):

I quite like this paper from Turn et al. and I think it should be published promptly in MBoC. The role of ElmoD proteins is understudied and this paper fills an important blank spot. I make some small suggestions to improve the work, but these are minor.

The paper begins with production of mutant MEFs lacking the function of the Arf GAPs Elmo1 and Elmo3 using CRISPR and a qualitative analysis of many aspects of cell biology that are unaltered, an important issue given the broad range of ARF effects. In contrast to their earlier study of Elmo2, the loss of either elmo1 or elmo3 led to a reduction in the number of ciliated cells. Importantly, re-expression of the gene rescues this phenotype, thus validating their mutants. Additional work reveals loss of a specific subset of ciliary proteins from the mutant cilia and an interesting accumulation of such protein in the Golgi. Finally, functional interactions with Arf3 and Arf16, but not Arf1 and Arf5 are demonstrated. On the whole the data are convincing, and the paper is very useful contribution. I strongly support publication.

Minor comments:

1. I understand that that Elmo1 or Elmo3 cannot be localized using immunostaining, but can it be detected by western blot in MEFs? This would be good to know so a comparison might be made to levels of expression of Eldm1/3-myc constructs.

2. Related: The authors say they scored only Myc-positive cells for rescues, but none are shown. Where is the myc signal in these cells? This should be shown.

3. I generally do not like graphs of subjective measures, such as cilia with reduced/normal/absent Arl13b. At a minimum this result must be scored blindly and a statement to that effect provided in the legend. Much, much better - both for objectivity and for the stats - is to simply use Fiji to measure average pixel intensity of arl13b in the cilium, which can easily be done using the acetylated tubulin to normalize the result.

4. I understand that scoring the golgi accumulation phenotype is more complex that the cilia phenotype I discussed above, so in this case blind scoring is essential.

5. I thought the Introduction and Discussion were too long (>150 citations!). It's well-written and I applaud their scholarship, but I feel that readers of THIS paper would be better served by a shorter, more focused Intro and Discussion. Then consider writing a review on this important topic!

Reviewer #2 (Remarks to the Author):

The ARF GAPs ELMOD1 and ELMOD3 act at the Golgi and Cilia to Regulate Ciliogenesis and Ciliary Protein Traffic

Rachel E. Turn, Yihan Hua, Skylar I. Dewees, Narra Devi, Michael P. East, Katherine R. Hardin, Tala Khatib, Joshua Linnert, Uwe Wolffman, Michael J. Lim, James E. Casanova, Tamara Caspary, and Richard A. Kahn*

The work by Turn et al., sheds new light on the functions of two highly conserved members of the ARF GAP family in ciliary trafficking, and at the same time reveals unexpected and new layers of complexity in the regulation of ciliary cargo transport. The study centers on ELMOD1 and ELMOD3, members of a family of three mammalian paralogs with GAP activity towards ARF and ARL GTPases, whose functions have not been previously characterized since their discovery by the Kahn lab and a link of ELMOD1 to autism spectrum disorder first described in 2019. Thus, this carefully controlled and well-executed cell and molecular focused study is timely and identifies ELMOD1 and ELMOD3 as having distinct functions in regulating ciliogenesis with opposing activities to those of the previously characterized ELMOD2 protein (Table I and summary Figure 6 provide excellent summaries). The work further pinpoints function at late licensing steps in ciliogenesis and roles in ciliary cargo trafficking that in the case of mutation significantly alters a subset of ciliary protein constituents (Arl3, Arl13b, INPP5E, IFT140). While the complexity of the biology raises new questions, the studies are thorough and lay important foundations for future work that make it important and of interest to the readership of MBoC.

Major recommendations center on:

1) text edits to ensure that the manuscript meets all of the stipulations on the Author Submission Checklist with regard to Data Presentation (list N = # in legends; additional detail re statistical tests used), Methodology and Statistics (statistical basis for choosing to analyze 100 cells); Reagents and Model Systems (e.g. reagent sharing, cell authentication, animal sex, age).

2) The introduction seems more like a general introduction drawn from a dissertation and would benefit from more succinctly describing the gaps in knowledge and relevance to the broader scope of science.
3) The discussion also would benefit from a more succinct summary of the key take home points.

Minor corrections/clarifications needed:

1. Abstract: Change "We believe..." to a statement that is factually supported

2. Results:

"We predict that all 17 lines result in the loss of functional proteins or null alleles, as these frameshifts are targeted upstream of the sole functional (ELMOD) domain." This statement raises the question as to why the authors did not look for confirmation by western blot. Only much later in the manuscript is it explained that there are no good antibodies and RNA and protein expression based on mass spec analyses are below detection. This information should be provided up front in the results section.

"There, in the basal part of the outer segment, the de novo formation of the photo-sensitive disc membranes of the outer segment occurs. This process is highly regulated by cilia-specific molecules and is powered by actin polymerization (95-97)." Clarification is needed on how these sentences are connected to ELMOD functions.

"We compared the relative levels of ELMOD1-myc and ELMOD3-myc expression by immunoblotting for the myc tag in total cell lysates of WT cells 24 hr after transient expression of each protein (Fig. S5). This revealed that ELMOD1-myc is expressed to considerably higher levels than is ELMOD3-myc (Fig. S5), perhaps explaining its greater potency in rescue." It is inferred just prior to this sentence that ELMOD1 and ELMOD3 might have different functions in ciliation, yet the sentence immediately thereafter suggests it could just be due to differences in the myc tagged protein levels. Clarify or refer to the discussion where this point is further discussed.

Explain the significance of differences in protein detection with different fixation methods, e.g., with respect to potential differences in lipid and cytoskeletal associations.

"In marked contrast, we did not detect any increased staining for other IFT proteins at the Golgi, including IFT81, IFT88, or IFT144 (data not shown)." Worth adding this data for the record?

Change KO'd to KO
Dear Dr. Spang,

On behalf of all authors, I would like to thank you and both reviewers for their time and efforts in reviewing and providing helpful critiques for our manuscript, "The ARF GAPs ELMOD1 and ELMOD3 act at the Golgi and Cilia to Regulate Ciliogenesis and Ciliary Protein Traffic." We are thrilled that both reviewers were so positive in their comments and are happy to address each point raised in our comments below and through edits to the text, as seen in the manuscript with edits highlighted by use of Track Changes. Below we have copied the entirety of the reviews received, followed after each point by our response(s). We have performed some additional analyses in an effort to completely address any issues raised and hope the manuscript is now acceptable to all.

Reviewer #1 (Remarks to the Author):
I quite like this paper from Turn et al. and I think it should be published promptly in MBoC. The role of ElmoD proteins is understudied and this paper fills an important blank spot. I make some small suggestions to improve the work, but these are minor.

The paper begins with production of mutant mefs lacking the function of the Arf GAPs Elmo1 and Elmo3 using CRISPR and a qualitative analysis of many aspects of cell biology that are unaltered, an important issue given the broad range of ARF effects. In contrast to their earlier study of Elmo2, the loss of either elmo1 or elmo3 led to a reduction in the number of ciliated cells. Importantly, re-expression of the gene rescues this phenotype, thus validating their mutants. Additional work reveals loss of a specific subset of ciliary proteins from the mutant cilia and an interesting accumulation of such protein in the golgi. Finally, functional interactions with Arf3 and Arf16, but not Arf1 and Arf5 are demonstrated. On the whole the data are convincing, and the paper is a very useful contribution. I strongly support publication.

Minor comments:
1. I understand that that Elmod1 or Elmod3 cannot be localized using immunostaining, but can it be detected by western blot in MEFs? This would be good to know so a comparison might be made to levels of expression of Eldm1/3-myc constructs.

   No, unfortunately, the endogenous ELMODs are not detectible by Western blotting of MEF lysates. This information has been added to the Results. Indeed, we had hoped to be able to compare endogenous to over-expressed proteins, but this is currently not doable.

2. Related: The authors say they scored only Myc-positive cells for rescues, but none are shown. Where is the myc signal in these cells? This should be shown.

   We have added a new supplementary figure (S10) just to show what the myc-tagged versions of ELMOD1 and ELMOD3 look like in MEFs. Note that the consistent result is evidence of staining at the plasma membrane, while the staining at internal structures or perhaps focal adhesions (in the case of ELMOD3-myc) is much more variable.

3. I generally do not like graphs of subjective measures, such as cilia with reduced/normal/absent Arl13b. At a minimum this result must be scored blindly and a statement to that effect provided in the legend. Much, much better - both for objectivity and for the stats- is to simply use Fiji to measure average pixel intensity of arl13b in the cilium, which can easily be done using the acetylated tubulin to normalize the result.

   Despite the fact that the differences in ARL13B staining intensities were quite obvious to the naked eye, we have now quantified these differences and added a new figure to supplementary (S11). We have also included statements confirming that scoring of all staining is
performed blindly.

4. I understand that scoring the golgi accumulation phenotype is more complex that the cilia phenotype I discussed above, so in this case blind scoring is essential.

   Scoring of all phenotypes is performed blinded, as now specifically stated in more than one place in the Methods section.

5. I thought the Introduction and Discussion were too long (>150 citations!). It's well-written and I applaud their scholarship, but I feel that readers of THIS paper would be better served by a shorter, more focused Intro and Discussion. Then consider writing a review on this important topic!

   We appreciate the concern, voiced by both reviewers, that the Introduction and Discussion are long. We have edited each section with an eye towards condensing yet retaining key concepts. However, a goal of this manuscript is that it be of interest and readily digestible to researchers focused on GTPases, cilia, membrane traffic, and lipid metabolism, and as a result we thought important to introduce more background for each of the key approaches and observations in the Introduction and perhaps more context than reviewers are used to in the Discussion. We hope a reasonable compromise has been reached. If more cutting is needed in the Discussion, we can delete the final paragraph. This was an attempt to highlight the limitations of the current study. We find this to be a growing and much needed trend that we encourage and support.

   We also went through the manuscript with a goal of reducing the number of citations and did end up deleting almost 50 of them. Given that we cover so much ground between explaining and discussing effects on ciliation, licensing, Golgi, membrane traffic, GTPases/GEFs/GAPs, lipid metabolism (Inpp5e), there are just going to be more references than usual. We hope this is agreeable to all.

**Reviewer #2 (Remarks to the Author):**
The work by Turn et al., sheds new light on the functions of two highly conserved members of the ARF GAP family in ciliary trafficking, and at the same time reveals unexpected and new layers of complexity in the regulation of ciliary cargo transport. The study centers on ELMOD1 and ELMOD3, members of a family of three mammalian paralogs with GAP activity towards ARF and ARL GTPases, whose functions have not been previously characterized since their discovery by the Kahn lab and a link of ELMOD1 to autism spectrum disorder first described in 2019. Thus, this carefully controlled and well-executed cell and molecular focused study is timely and identifies ELMOD1 and ELMOD3 as having distinct functions in regulating ciliogenesis with opposing activities to those of the previously characterized ELMOD2 protein (Table I and summary Figure 6 provide excellent summaries). The work further pinpoints function at late licensing steps in ciliogenesis and roles in ciliary cargo trafficking that in the case of mutation significantly alters a subset of ciliary protein constituents (Arl3, Arl13b, INPP5E, IFT140). While the complexity of the biology raises new questions, the studies are thorough and lay important foundations for future work that make it important and of interest to the readership of MBoC.

Major recommendations center on:

1) text edits to ensure that the manuscript meets all of the stipulations on the Author Submission Checklist with regard to Data Presentation (list N = # in legends; additional detail re statistical
tests used), Methodology and Statistics (statistical basis for choosing to analyze 100 cells); Reagents and Model Systems (e.g. reagent sharing, cell authentication, animal sex, age).

We went back through the text with an eye for meeting the Author Submission Checklist, with particular focus on fixing data presentation and statistics. There was a late change in how data were displayed in several figures that were not accompanied by the needed changes in figure legends in our original submission. This has now been corrected and we thank you for catching it. Although counting 100 cells is the standard in the field and is used in countless papers, we also performed a power analysis to confirm that we are over powered in scoring 100 cells in our assays.

2) The introduction seems more like a general introduction drawn from a dissertation and would benefit from more succinctly describing the gaps in knowledge and relevance to the broader scope of science.

See comments to reviewer #1, point 5, above.

3) The discussion also would benefit from a more succinct summary of the key take home points.

See comments to reviewer #1, point 5, above.

Minor corrections/clarifications needed:

1. Abstract: Change "We believe..." to a statement that is factually supported

   Wording in the Abstract has been changed along the lines requested, highlighting our model that is supported by the bulk of the data presented. Thus, in stead of “Thus, we believe that ELMOD1...” it now reads, “Thus, our data support a model in which ELMOD1...”

2. Results:

"We predict that all 17 lines result in the loss of functional proteins or null alleles, as these frameshifts are targeted upstream of the sole functional (ELMOD) domain." This statement raises the question as to why the authors did not look for confirmation by western blot. Only much later in the manuscript is it explained that there are no good antibodies and RNA and protein expression based on mass spec analyses are below detection. This information should be provided up front in the results section.

   The requested clarification has been added to the first paragraph of the Results section, immediately after the quoted line. Thus:
   “We predict that all 17 lines result in the loss of functional proteins or null alleles, as these frameshifts are targeted upstream of the sole functional (ELMOD) domain. Thus, we refer to these lines as knockouts (KOs) and double KO (Elmod1/Elmod3 DKO) lines. There are currently no antibodies specific to ELMOD1 or ELMOD3 with requisite sensitivity to detect endogenous proteins in MEFs, so further confirmation by immunoblotting was not possible.”

"There, in the basal part of the outer segment, the de novo formation of the photo-sensitive disc membranes of the outer segment occurs. This process is highly regulated by cilia-specific molecules and is powered by actin polymerization (95-97).” Clarification is needed on how these sentences are connected to ELMOD functions.
This was intended as additional information for those less up on cilia/outer segment formation and function(s), but this reviewer is correct that it is not particularly germane to how we model ELMOD1/3 to be acting. So we have deleted these sentences.

"We compared the relative levels of ELMOD1-myc and ELMOD3-myc expression by immunoblotting for the myc tag in total cell lysates of WT cells 24 hr after transient expression of each protein (Fig. S5). This revealed that ELMOD1-myc is expressed to considerably higher levels than is ELMOD3-myc (Fig. S5), perhaps explaining its greater potency in rescue." It is inferred just prior to this sentence that ELMOD1 and ELMOD3 might have different functions in ciliation, yet the sentence immediately thereafter suggests it could just be due to differences in the myc tagged protein levels. Clarify or refer to the discussion where this point is further discussed.

Unfortunately, our inability to assess endogenous levels of ELMOD1 or 3 and differences in their levels of expression from plasmids (despite expression from the same vector and tagging!) results in complications in interpretation of some of our findings. In an effort to inform the reader as to why we believe these data are important and how we interpret them (and to send them to the Discussion for more) we added a sentence after the cited ones: “We currently interpret these findings as more consistent with ELMOD1 and ELMOD3 acting on a common pathway but at distinct steps, as explained in more detail in the Discussion.”

Explain the significance of differences in protein detection with different fixation methods, e.g., with respect to potential differences in lipid and cytoskeletal associations.

We are not sure we understand the ask here but have tried to address it by adding a few sentences to the Methods, given the desire to limit the length of Introduction and Discussion and it doesn’t seem appropriate in Results. The uncertainty on our part comes from the belief that researchers who use indirect immunofluorescence should be aware of potentially large differences in staining, depending upon the methods used for fixation and permeabilization.

Under the section “Immunofluorescence” in Methods we have added the following: “Variations in staining of cells after different fixation or permeabilization protocols using one antibody typically arise as a result of changes in protein structure and epitope exposure, retention/loss of binding partners that also may alter epitope availability, or washout of one pool of antigen that may have masked the staining of another pool. The last is common for proteins that are largely soluble but with transient localization to membrane compartments, as is common for ARF family GTPases and their regulators.”

"In marked contrast, we did not detect any increased staining for other IFT proteins at the Golgi, including IFT81, IFT88, or IFT144 (data not shown)." Worth adding this data for the record?

We had discussed this point before the original submission and again after receiving these reviews. We conclude that the data in question would simply appear as Golgi marker along with lack of staining for other antigens. We find no real value in including such data and opt to continue to not show such negative data. We hope the current 9 supplementary figures, many of them multi-panels, will suffice.

Change KO'd to KO
Done.
Dear Dr. Kahn:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Anne Spang
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Kahn:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

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We are pleased that you chose to publish your work in MBoC.

Sincerely,
Eric Baker
Journal Production Manager
MBoC Editorial Office
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