Roles for ELMOD2 and Rootletin in Ciliogenesis

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RE: Manuscript #E20-10-0635
TITLE: Roles for ELMOD2 and Rootletin in Ciliogenesis

Dear Dr. Kahn:

Your manuscript has now been seen by two expert reviewers whose comments are enclosed. As you will see they both agree that your study is interesting and potentially important and could appeal to the broad readership of MBoC. However, they raise a number of points that will require clarification, and in some instances further experiments and data analysis, before the manuscript can be accepted for publication.

The reviewers share a major concern that the pleiotropic nature of ELMOD2 makes it difficult to conclude that the cilia phenotypes of the null cells are a direct consequence of loss of centrosomal ELMOD2. In particular, ELMOD2-deficient cells exhibit polyploidisation, cell cycle abnormalities and centrosome amplification, each of which can disrupt ciliogenesis in an indirect manner. It is therefore essential that your revised manuscript addresses this issue along the lines suggested by the reviewers such as including only those cells in the analyses that contain normal centriole numbers. The rescue experiments are promising, but I agree with the reviewer that examination of polyploidy, a possible confounding factor, should be carried out in these instances.

When submitting your revised manuscript, please respond to all the points made by the reviewers. If you do not agree with any, please explain why. To speed up the review process, my intention is to return the revision to the same reviewers.

I do hope you will be able to make the recommended changes as we would like to publish your work.

Sincerely,

Fanni Gergely
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Kahn,

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.

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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 study by Turn and colleagues investigates the centriolar, rootlet and ciliary functions of the GTPase activating protein ELMOD2. The authors demonstrate that deletion of ELMOD1 enhanced ciliary assembly, caused an increase in the number of centrioles, and the formation of excess cilia (multiciliation). Loss of ELMOD2 resulted in changes in ciliary protein content (both increases and decreases in certain components), suggesting a role for this protein in regulating the composition of ciliary proteins. They show that ELMOD2 localizes to centriolar rootlets and cilia in a number of cell types, including fibroblasts and photoreceptor cells. Loss of ELMOD2 caused fragmentation of centriolar rootlets and defects in centrosome cohesion. Consistent with this, deletion of rootletin resulted in loss of ELMOD2 at basal bodies and resulted in similar phenotypic defects as loss of ELMOD2. Finally, they suggest that ELMOD2 and rootletin cooperate to regulate the ciliogenesis by preventing spurious licensing through release of the centriole capping protein CP110.

My main concern with this manuscript is that many of the observed centriolar, rootlet and ciliary phenotypes could be an indirect consequence of the many roles that ELMOD2 plays in various cellular compartments (lipid droplets, ER, mitochondria, midbodies etc), and the number of other cellular defects that have been described previously (cytokinesis defects, polyploidy, microtubule nucleation and stability etc). As shown in Figure S7 (in addition to other publications) the vast majority of ELMOD2 is NOT at the centrosome nor cilium. Although the localization of this protein to rootlets is fairly convincing (particularly since the staining is also seen in photoreceptors), it is difficult for this reviewer to believe that the ciliary phenotypes observed upon loss of ELMOD2 are specifically due to the pool at the rootlets/cilia. This is something that the authors themselves acknowledge in the Discussion section, and point to results from the GAP-dead version as supportive proof that is does. However, I remain somewhat skeptical that all of these phenotypes are directly related to its function at rootlets, which tempers my enthusiasm for this study.

Specific concerns:

Fig 1 A-C: ELMOD2 KO causes enhanced ciliation, even in the presence of serum. Could this be due to cell-cycle arrest, or quiescence, which would promote cilia formation?

As the authors indicate, ELMOD2 KO lines show polyploidy. Thus, one reason why the localization of rootlets and ciliary proteins are altered in the mutant cells could be due to changes in the expression levels of these genes. For example, a recent study of Trisomy-21 cells (Galati et al, Dev Cell, 2018), where there is an increase in only one chromosome (compared to the many in a polyploid cell), results in increased expression of pericentrin (since there is an extra copy of the gene). The changes in expression of that one protein alone caused significant alterations in centrosome composition, microtubule nucleation centrosome protein trafficking... resulting in many of the same ciliary protein concentration defects observed here (for example, Shh signaling). Therefore, how can the authors be sure that what they are noting as changes in protein localization is not merely caused by changes in protein expression, due to changes in chromosome number? I appreciate that rescue experiments were performed - but how do the authors think that re-introducing ELMOD2 in KO lines rescues cellular defects such as polyploidy (that already is manifested in the KO lines)?

One potential way to address these changes would be to perform immunoblotting of total protein lysates from Wt and ELMOD2 KO cells for the various components studied here.

Similarly, Fig 3 B shows dramatic changes in rootletin levels and localization in ELMOD2 mutant cells... but those cells are clearly grossly abnormal, with gigantic polyploidy nuclei compared to the controls. Have the authors quantified the relative abundance of rootletin at centrosomes of ELMOD2-null cells that are more "normal" in morphology (i.e. without cytokinesis defects, or multinucleation/polyploidy)?

Fig 8 - the quantification of the fraction of centrosomes with Cep164, TTBK2 and CP110 is a bit confusing, making interpretation of this result difficult. The authors suggest that ELMOD2 loss directly causes licensing of ciliogenesis by causing acquisition of Cep164 and loss of CP110. However, this again could be an indirect consequence. Since roughly a third of the ELMOD2-null cells contain excess centrosomes, it would be expected that a number of these would become mature, mother centrioles... since passage through the cell cycle (specifically mitosis) allows a fraction of daughter centrioles to mature, acquire

Cep164 and lose CP110 (which is consistent with the multiciliation observed). This happens in most cell types displaying centrosome amplification, independent of ELMOD2 loss. A key question then is: in ELMOD2-null cells that only contain the normal complement of centrioles (1 mother, 1 daughter), is CP110 lost from the daughter centriole? Does it acquire Cep164? One way to test this is to look in serum starved ELMOD2 KO cells, but focus only on cells with 2 centrioles (not the ones with centrosome amplification). If ELMOD2 is involved in this licensing INDEPENDENTLY of cell-cycle mediated maturation of daughter centrioles, then the authors can make the conclusion that it is indeed involved in licensing.

Minor points:

The authors refer to the ELMOD2 localization pattern at centrioles as "foot-like" structures. This could be confusing, since certain centrioles contain structures called "basal feet". I believe rootlets are commonly referred to as striated fibers... so the authors may want to stick with that nomenclature.

Missing scale bars in Fig 1H, S2, S4, S11C

Reviewer #2 (Remarks to the Author):

Turn, Kahn and colleagues here describe how the GTPase activating protein, ELMOD2, localises to ciliary rootlet structures in mouse embryonic fibroblasts and in human and mouse retinal tissues. They demonstrate that ELMOD2 deletion causes increased and aberrant primary ciliogenesis, along with disruption of the rootlet and increased centrosome separation. They show that genome editing-mediated removal of Rootletin, a known component of the ciliary rootlet, increases centrosome separation and ciliation frequency, while disrupting ELMOD2 localisation to rootlets. Expression of an N-terminal deletion form of Rootletin blocks ciliogenesis, but does not impact on centrosome separation. Rescue experiments indicate that wild-type Rootletin overexpression can rescue the ciliation and centrosome separation phenotypes of both the ELMOD2 and Rootletin knockouts. Exploration of the roles of the GTPase functions demonstrated that ELMOD2's GTPase activating functions were dispensable in rescuing the ciliation phenotypes seen in the ELMOD2 nulls, and that the GTPase ARL2 was the key activity directed to rootlets by ELMOD2 and Rootletin to regulate ciliogenesis. The authors report that ELMOD2 and Rootletin deficient cells show abnormal frequencies of CEP164 localization to centrioles, along with CP110 loss from centrioles, leading to a model where ELMOD2 and Rootletin control the ciliary rootlet in regulating key early steps in primary ciliogenesis.

There is a strong dataset here that presents a potentially exciting finding, of the ciliary rootlet playing a regulatory role in primary ciliogenesis. These findings will be of general interest. However, the results are not yet convincing and require additional controls. There are other potential mechanisms that could affect the observations presented here and that should be controlled for.

1. A significant potential confounding factor is the number of centrosomes per cell and the separation (or not) of individual centrioles. The ELMOD2 cells are polyploid and multinucleated, from the authors' recent study (Turn et al. (2020) MBoC 31:2070-2091). Multiple or separated centrioles generated by PLK4 overexpression (Mahjoub et al. (2012) Curr. Biol. 22:1628-1634) or irradiation (Conroy et al. (2012) Cell Cycle 11:3769-3778) also support multiple cilia, with some aberrant structures also having been observed in these studies. These points should be controlled for, so that a cell with multiple centrosomes (which may have multiple mother centrioles) should not be compared with a cell that contains only one centriole pair. Restricting the analysis to cells with only 2 or 4 centrioles and stratifying these as G0/G1 or S/G2 phase centriole pairs would be one means of addressing this concern (although this does not consider the possibility of G1 cells with 4 centrioles in the ELMOD2 KOs).

2. A difficulty in establishing a model for the rootlet (or centrosome cohesion) in ciliary regulation is the lack of ciliary phenotype in C-NAP1-deficient cells, which lose the rootlet attachment to the centrosomes, but do not show an abnormal ciliary frequency (as reported by Graser et al. (2007) JCB 179: 321-330; Panic et al. (2015) PLoS Genet 11(5): e1005243; Mazo et al. (2016) Dev Cell 39: 1-14; Flanagan et al. (2017) MBoC 28:736-745). It would be useful for the authors to discuss this point.

3. Related to point 2., the authors should determine in multiciliated ELMOD2 (and Rootletin) knockout cells whether the cilia arise from individual or paired centrioles, i.e., is the centrosome separation a potential factor? The point here is whether the younger mother provides the basal body, or whether multiple, daughter centrioles now become capable of allowing ciliation.

4. Cell cycle analysis of the Rootletin KO cells should be performed, to assess whether there are any defects in their cell cycle distributions that might cause polyploidy or changes in centrosome numbers that could impact the analyses here.

5. A quantitation of the centrin signals seen in Fig S1 is necessary to support the authors' suggestion of a specific alteration in intraciliary transport brought about by ELMOD2 loss. There is an inconsistency in the staining for centrin that is of concern: Fig S1 shows cells with marked centrin background staining, as well as cells without (with no correlation with ELMOD2 or ciliation status). This suggests that the staining/ exposure necessary to visualise centrin may be variable, which may confound the putative ciliary localisation described here

6. Figure S3 should include some quantitation of the data for SSTR3 and GPR161 localisation.

7. Quantitation of the cells with ELMOD2 signal after ciliobrevin treatment should be provided for Fig S2. The KO control should be shown.

8. Some clarification of the reproducibility of the dynamic ELMOD2 staining at rootlet structures should be provided. As presented, the observations are highly qualitative.

9. A control should be provided for the overexpression of ELMOD2 in the Rootletin KO cell rescue experiment. Similarly, expression controls should be provided for the activated ARL2, ARL3, ARL6, ARF6 and ARL13B transient overexpression experiments.

10. The imaging that provides the basis for the quantitations in Figure 8 should be shown.

11. The proposed fragmentation of rootlets in the ELMOD2 knockouts is unclear- are rootlets still associated with (multiple) centriole proximal ends or are there multiple aggregates of Rootletin?

12. The levels of Rootletin should be evaluated in ELMOD2 KO cells (this seems to be same as wild-type in Figure 4A, but this is a tangential observation there and should be tested specifically).

13. It is unclear what is being examined in Fig 3F, particularly in the ELMOD2 KO sample. The imaging of gamma tubulin should be improved to visualise individual structures; showing fewer cells might help with this aspect. It is also unclear whether the authors are looking at individual centrioles (not quantitated) or what they score as 'centrosomes' in their separation assay. This should be clarified.

14. The data for Cep44 shown in Figure S12 are not clear. There appear to be qualitative and quantitative differences in the Cep44 labelling in the various mutant cells, so this experiment should be reviewed. Quantitation of the analysis would allow a more convincing conclusion to be drawn regarding Cep44.

Minor points

15. A general point: placing the blow-ups of parts of the image within the larger image negates the purpose of having more cells in the field. Several of the images selected have redundant elements in them that contribute little and make interpretation difficult for the reader (e.g. Figs. 2A and 2D, Fig. 3F). For example, the Rootletin KO image in Fig. 4F shows a cell at right that is greatly obscured by the blow-up. The delta239 image fails to show the whole of the cell of interest. This is not ideal and the authors might help readers by simplifying their presentation.

16. Supplemental Figures should be cited in order in the text.

17. The centrin antibody used should be specified as clone 20H5.

18. Figure 4 is cited instead of Figure 7 on p10, 11; this should be corrected.

19. Have the authors any data on what happens with expression of Rootletin 1-239? I do not suggest this as a required experiment, but it stands out as an attractive potential addition, if such data were available.

Dear Dr. Gergely,

We appreciate the time, efforts, and insights provided by both the reviewers and the editor in the review of our manuscript. The feedback provided has helped us to strengthen our story, shedding novel insights into ELMOD2's function as a regulator of ciliogenesis, rootlets, and centrosome cohesion. With additional, new experiments addressing aspects of our study, we have shown that increased ciliation, multiciliation, and recruitment of ciliogenesis factors are even more clearly independent of cell cycle. We have also highlighted that though deletion of ELMOD2 causes defective cytokinesis, Rootletin null lines reveal no such cytokinesis defects (i.e multinucleation, supernumerary centrosomes). We also believe that the reformatting of figures, the additional control studies, and the more detailed writing suggested by reviewers have strengthened our story overall and made it acceptable by all reviewers and editors.

We respond to issues raised in your cover letter here and below have pasted the entirety of the two reviews, along with our detailed responses under each point. While we do not agree with every issue raised, we explain and have made substantial changes in line with recommendations and performed considerable new experimentation, resulting in what we believe to be a stronger model. We hope and trust that all will now find it acceptable for publication.

Rick Kahn, for all authors

Dear Dr. Kahn:

Your manuscript has now been seen by two expert reviewers whose comments are enclosed. As you will see they both agree that your study is interesting and potentially important and could appeal to the broad readership of MBoC. However, they raise a number of points that will require clarification, and in some instances further experiments and data analysis, before the manuscript can be accepted for publication.

The reviewers share a major concern that the pleiotropic nature of ELMOD2 makes it difficult to conclude that the cilia phenotypes of the null cells are a direct consequence of loss of centrosomal ELMOD2. In particular, ELMOD2-deficient cells exhibit polyploidisation, cell cycle abnormalities and centrosome amplification, each of which can disrupt ciliogenesis in an indirect manner. It is therefore essential that your revised manuscript addresses this issue along the lines suggested by the reviewers such as including only those cells in the analyses that contain normal centriole numbers. The rescue experiments are promising, but I agree with the reviewer that examination of polyploidy, a possible confounding factor, should be carried out in these instances.

We completely agree with this concern, and for that reason the second paragraph of the Discussion was intended to address it head on. Clearly, our earlier attempt was not sufficient so we have performed additional experiments to try to persuade all readers of the distinct nature of the cell cycle and ciliation phenotypes. That said, it is important for all to bear in mind that such processes are in fact inter-connected so complete resolution is impractical at this time, or at least until a far more complete understanding of the molecular details of each are in hand. Although ciliogenesis and cell cycle certainly have at least some degree of crosstalk, we believe that ELMOD2 is performing discrete functions in each pathway and that defective cytokinesis clearly is not sufficient to explain the ciliary phenotypes described in our study. First, ELMOD2 KO cells have a specific defect/stalling in the final stages of cell division, abscission. Such cells

are incapable of forming cilia, and therefore we would have expected, if anything, for cells to be incapable of ciliogenesis. Instead, we see the opposite: increased ciliation, multiciliation, and abnormal ciliary morphology/protein content. This alone makes it unlikely that ELMOD2's actions in the cell cycle could explain these defects in ciliation. Our studies also reveal that only the activated mutant of ARL2 can reverse the increased ciliogenesis observed in ELMOD2 KO cells. If it was ELMOD2's function in cell cycle that was driving the ciliogenesis defects, we would have expected ARF6 to reverse the defect, as we previously reported ELMOD2 is working through ARF6 to drive cytokinesis. To fully test this model and to strengthen this story, we followed the advice of the reviewers and performed a number of experiments to address these points: scoring multiciliation only in "normal" cells (e.g. mononucleated, 1-2 centrosomes), scoring TTBK2 recruitment in "normal" cells, and performing flow cytometry of Rootletin KO cells to check for cell cycle defects. We are grateful for the advice that we received, and we believe these experiments have helped us make a stronger story and even more clearly separate these two central roles for ELMOD2 in mammalian cells.

When submitting your revised manuscript, please respond to all the points made by the reviewers. If you do not agree with any, please explain why. To speed up the review process, my intention is to return the revision to the same reviewers.

I do hope you will be able to make the recommended changes as we would like to publish your work.

Thank you for this opportunity to re-submit. We are taking you up on this offer here and encourage you to send to these and/or other reviewers as needed to ensure that our best efforts are evident. That said, we have made every effort to respond as completely as possible to the issues raised by reviewers and hope that we might get a positive response before the next grant submission deadline of January 19th, 2021, if at all possible.

Sincerely,

Fanni Gergely
Monitoring Editor
Molecular Biology of the Cell

Reviewer #1 (Remarks to the Author):

This study by Turn and colleagues investigates the centriolar, rootlet and ciliary functions of the GTPase activating protein ELMOD2. The authors demonstrate that deletion of ELMOD1 enhanced ciliary assembly, caused an increase in the number of centrioles, and the formation of excess cilia (multiciliation). Loss of ELMOD2 resulted in changes in ciliary protein content (both increases and decreases in certain components), suggesting a role for this protein in regulating the composition of ciliary proteins. They show that ELMOD2 localizes to centriolar rootlets and cilia in a number of cell types, including fibroblasts and photoreceptor cells. Loss of ELMOD2 caused fragmentation of centriolar rootlets and defects in centrosome cohesion. Consistent with this, deletion of rootletin resulted in loss of ELMOD2 at basal bodies and resulted in similar phenotypic defects as loss of ELMOD2. Finally, they suggest that ELMOD2 and rootletin cooperate to regulate the ciliogenesis by preventing spurious licensing through release of the centriole capping protein CP110.

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cellular compartments (lipid droplets, ER, mitochondria, midbodies etc), and the number of other cellular defects that have been described previously (cytokinesis defects, polyploidy, microtubule nucleation and stability etc). As shown in Figure S7 (in addition to other publications) the vast majority of ELMOD2 is NOT at the centrosome nor cilium. Although the localization of this protein to rootlets is fairly convincing (particularly since the staining is also seen in photoreceptors), it is difficult for this reviewer to believe that the ciliary phenotypes observed upon loss of ELMOD2 are specifically due to the pool at the rootlets/cilia. This is something that the authors themselves acknowledge in the Discussion section, and point to results from the GAP-dead version as supportive proof that is does. However, I remain somewhat skeptical that all of these phenotypes are directly related to its function at rootlets, which tempers my enthusiasm for this study.

Specific concerns:
Fig 1 A-C: ELMOD2 KO causes enhanced ciliation, even in the presence of serum. Could this be due to cell-cycle arrest, or quiescence, which would promote cilia formation?

First a general comment. After working on ARF family GTPases and their regulators for several decades, it is fair to say that it is very rare indeed that the majority of any of these proteins is at any one site of action, so no one should be surprised to see strong phenotypes at one location when there is lots of the targeted protein at other locations. The prototype here is perhaps ARF1, which clearly acts at the Golgi, yet at any one time probably less than 10% is on Golgi membranes as it must be transiently (!) recruited from cytosol. This has also been shown quantitatively for RAS proteins, as there is an antibody that binds only the activated species. But now back to the point at hand. We believe that it is unlikely that quiescence/cell cycle arrest would explain these results, specifically because of the nature of the cell cycle defect that ELMOD2 KO cells have. They are locked in cytokinesis/G2/M, as we've reported previously. Typically, cells undergoing division have resorbed their cilia, and the fact that these cells are stuck in that particular stage of cell cycle rather than G1 makes it unlikely that this would reveal itself with INCREASED ciliation, multiciliation, and ciliary traffic defects. We have added text to the Discussion in efforts to better clarify these important issues.

As the authors indicate, ELMOD2 KO lines show polyploidy. Thus, one reason why the localization of rootlets and ciliary proteins are altered in the mutant cells could be due to changes in the expression levels of these genes. For example, a recent study of Trisomy-21 cells (Galati et al, Dev Cell, 2018), where there is an increase in only one chromosome (compared to the many in a polyploid cell), results in increased expression of pericentrin (since there is an extra copy of the gene). The changes in expression of that one protein alone caused significant alterations in centrosome composition, microtubule nucleation centrosome protein trafficking... resulting in many of the same ciliary protein concentration defects observed here (for example, Shh signaling). Therefore, how can the authors be sure that what they are noting as changes in protein localization is not merely caused by changes in protein expression, due to changes in chromosome number? I appreciate that rescue experiments were performed - but how do the authors think that re-introducing ELMOD2 in KO lines rescues cellular defects such as polyploidy (that already is manifested in the KO lines)?

We agree that the relationship between polyploidy and ciliation may well be more complicated, and the paper cited makes this point. That said, clearly and definitively resolving such things is viewed as beyond the scope of this manuscript. In a best attempt to address this issue, however, we have gone back and re-scored ciliation/multiciliation, this time only scoring those cells with a single, "normal" sized nucleus and no more than two centrosomes. The results of

such scoring are shown in Figure 1F, and further support our original conclusion. Thus, we thank the reviewer for the suggestion as it strengthens our claims.

One potential way to address these changes would be to perform immunoblotting of total protein lysates from Wt and ELMOD2 KO cells for the various components studied here.

This is a very fair point- that having increased copies of DNA could most definitely lead to increased (unknown) protein production and could have downstream effects on protein localization in the cell. We've addressed this by immunoblotting for three proteins: centrin, Rootletin, and ARL3 in WT vs KO lines. ELMOD2 KO cells do not show any clear changes in any of the three proteins listed here, so we feel more comfortable claiming that the changes in protein localization and increased ciliation are independent of cell cycle. Of course, we cannot be sure that some other change in protein abundance, coming from an extra chromosome, might not have such an effect, but at least the ones monitored don't appear to be responsible. Thank you for the suggestion- we believe this experiment helps strengthen our story. Please see Figure S14 and Figure S15 for details of these new data.

Similarly, Fig 3 B shows dramatic changes in rootletin levels and localization in ELMOD2 mutant cells... but those cells are clearly grossly abnormal, with gigantic polyploidy nuclei compared to the controls. Have the authors quantified the relative abundance of rootletin at centrosomes of ELMOD2-null cells that are more "normal" in morphology (i.e. without cytokinesis defects, or multinucleation/polyploidy)?

We apologize for the cell chosen for the "representative image"- ELMOD2 KO cells have gross nuclei because of their cell cycle defects, but one point of our paper is to highlight that cell cycle alone cannot explain the spurious ciliogenesis that we observe in ELMOD2 KO cells. Therefore, we've included a field of cells that are more "normal" in morphology to better highlight that Rootletin is abnormal even in cells with one, normal-sized nucleus, and absent centrosome amplification.

Fig 8 - the quantification of the fraction of centrosomes with Cep164, TTBK2 and CP110 is a bit confusing, making interpretation of this result difficult. The authors suggest that ELMOD2 loss directly causes licensing of ciliogenesis by causing acquisition of Cep164 and loss of CP110. However, this again could be an indirect consequence. Since roughly a third of the ELMOD2-null cells contain excess centrosomes, it would be expected that a number of these would become mature, mother centrioles... since passage through the cell cycle (specifically mitosis) allows a fraction of daughter centrioles to mature, acquire Cep164 and lose CP110 (which is consistent with the multiciliation observed). This happens in most cell types displaying centrosome amplification, independent of ELMOD2 loss. **A key question then is**: in ELMOD2-null cells that only contain the normal complement of centrioles (1 mother, 1 daughter), is CP110 lost from the daughter centriole? Does it acquire Cep164? One way to test this is to look in serum starved ELMOD2 KO cells, but focus only on cells with 2 centrioles (not the ones with centrosome amplification). If ELMOD2 is involved in this licensing INDEPENDENTLY of cell-cycle mediated maturation of daughter centrioles, then the authors can make the conclusion that it is indeed involved in licensing.

We agree that if ELMOD2 is truly acting as an inhibitor of spurious ciliogenesis, we would expect that we would see increased ciliogenesis marker recruitment even in cells having only one nucleus and no more than 2 centrosomes. Therefore, we have now re-scored TTBK2 recruitment to centrosomes only looking at such cells. The results continue to support our model. Please see Figure S16 for the data.

Minor points:
The authors refer to the ELMOD2 localization pattern at centrioles as "foot-like" structures. This could be confusing, since certain centrioles contain structures called "basal feet". I believe rootlets are commonly referred to as striated fibers... so the authors may want to stick with that nomenclature.

Thank you for bringing up this point- it could definitely bring about confusion! We have decided to change the word to "protrusion" to reduce confusion. We opted out of the use of the word "striated fiber" since the choice of the word "foot-like" was mainly for the purpose of describing gross morphology. In the timeline of the text, we did not yet know what exactly ELMOD2 was colocalizing with.

Missing scale bars in Fig 1H, S2, S4, S11C

Thanks for catching this. We've addressed this oversight and inserted scale bars in the indicated figures.

Reviewer #2 (Remarks to the Author):

Turn, Kahn and colleagues here describe how the GTPase activating protein, ELMOD2, localises to ciliary rootlet structures in mouse embryonic fibroblasts and in human and mouse retinal tissues. They demonstrate that ELMOD2 deletion causes increased and aberrant primary ciliogenesis, along with disruption of the rootlet and increased centrosome separation. They show that genome editing-mediated removal of Rootletin, a known component of the ciliary rootlet, increases centrosome separation and ciliation frequency, while disrupting ELMOD2 localisation to rootlets. Expression of an N-terminal deletion form of Rootletin blocks ciliogenesis, but does not impact on centrosome separation. Rescue experiments indicate that wild-type Rootletin overexpression can rescue the ciliation and centrosome separation phenotypes of both the ELMOD2 and Rootletin knockouts. Exploration of the roles of the GTPase functions demonstrated that ELMOD2's GTPase activating functions were dispensable in rescuing the ciliation phenotypes seen in the ELMOD2 nulls, and that the GTPase ARL2 was the key activity directed to rootlets by ELMOD2 and Rootletin to regulate ciliogenesis. The authors report that ELMOD2 and Rootletin deficient cells show abnormal frequencies of CEP164 localization to centrioles, along with CP110 loss from centrioles, leading to a model where ELMOD2 and Rootletin control the ciliary rootlet in regulating key early steps in primary ciliogenesis.

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1. A significant potential confounding factor is the number of centrosomes per cell and the separation (or not) of individual centrioles. The ELMOD2 cells are polyploid and multinucleated, from the authors' recent study (Turn et al. (2020) MBoC 31:2070-2091). Multiple or separated centrioles generated by PLK4 overexpression (Mahjoub et al. (2012) Curr. Biol. 22:1628-1634) or irradiation (Conroy et al. (2012) Cell Cycle 11:3769-3778) also support multiple cilia, with some aberrant structures also having been observed in these studies. These points should be

controlled for, so that a cell with multiple centrosomes (which may have multiple mother centrioles) should not be compared with a cell that contains only one centriole pair. Restricting the analysis to cells with only 2 or 4 centrioles and stratifying these as G0/G1 or S/G2 phase centriole pairs would be one means of addressing this concern (although this does not consider the possibility of G1 cells with 4 centrioles in the ELMOD2 KOs).
Please see above comments to the specific concerns of Reviewer #1. These points will be explored further in discussion.

2. A difficulty in establishing a model for the rootlet (or centrosome cohesion) in ciliary regulation is the lack of ciliary phenotype in C-NAP1-deficient cells, which lose the rootlet attachment to the centrosomes, but do not show an abnormal ciliary frequency (as reported by Graser et al. (2007) JCB 179: 321-330; Panic et al. (2015) PLoS Genet 11(5): e1005243; Mazo et al. (2016) Dev Cell 39: 1-14; Flanagan et al. (2017) MBoC 28:736-745). It would be useful for the authors to discuss this point.

We agree that models of the roles of rootlets in centrosome cohesion and links to cilia currently are not all in agreement and well supported. For example, it is surprising to us that we see increased ciliogenesis and multiciliation in cells lacking Rootletin, while these phenotypes have not been observed by other groups. This may in part be explained by the fact that many of those studies are focusing instead upon the centrosome cohesion rather than exploring the relationship of centrosome cohesion and cilia (as seen in the Graser et al paper which focuses only on the cohesion). In fact, we have data from another project in our lab in which we again observe rootlet fragmentation yet no loss in centrosome cohesion. Thus, we believe that current models are not sufficient to explain the relationship between rootlets and cohesion and we hope to explore such questions in the near future but that they are beyond the scope of this current manuscript. We have modified our Discussion in efforts to clarify this point.

3. Related to point 2., the authors should determine in multiciliated ELMOD2 (and Rootletin) knockout cells whether the cilia arise from individual or paired centrioles, i.e., is the centrosome separation a potential factor? The point here is whether the younger mother provides the basal body, or whether multiple, daughter centrioles now become capable of allowing ciliation.

Indeed, this is an important and interesting point! From all our studies, the cilia of multiciliated cells each emanate from a single centrosome. We have not seen a cell in which two cilia emanate from the same centrosome (aka both the mother and daughter centrioles of a single centrosome are generating cilia). From our experience, there doesn't appear to be a trend in whether cilia come from paired centrioles or individual centrioles- it appears that multiciliation emerges under both conditions. Our Crocc null cells do not display centrosome amplification, further supporting the one centrosome:one cilium model. These observations will be covered more in Discussion.

4. Cell cycle analysis of the Rootletin KO cells should be performed, to assess whether there are any defects in their cell cycle distributions that might cause polyploidy or changes in centrosome numbers that could impact the analyses here.

This is a good control experiment because if we believe that ELMOD2 is regulating ciliogenesis independent of its cell cycle functions and also working through Rootletin, it would support our model if Rootletin did not show stalled cytokinesis but still displayed increased ciliogenesis and multiciliation. We performed these experiments and found no evidence of changes in cell cycle populations in log phase growing Rootletin KO compared to WT cells. Please see Figure S14.

5. A quantitation of the centrin signals seen in Fig S1 is necessary to support the authors' suggestion of a specific alteration in intraciliary transport brought about by ELMOD2 loss. There is an inconsistency in the staining for centrin that is of concern: Fig S1 shows cells with marked centrin background staining, as well as cells without (with no correlation with ELMOD2 or ciliation status). This suggests that the staining/ exposure necessary to visualise centrin may be variable, which may confound the putative ciliary localisation described here

We understand the concern based on our poor choice of the images used in Fig. S1. After reviewing the relevant slides/data to make sure we were not biasing our conclusions, we more carefully collected representative images with similar levels of background staining to better highlight the point of the images shown. Please see Figure S1 for the revision. We have found that aging of our secondary antibodies can lead to differences in background staining that we now pay much more attention to and control for between conditions. Though likely not the only cause of such variation, it is one easily controlled for now and in the future.

6. Figure S3 should include some quantitation of the data for SSTR3 and GPR161 localisation.

We addressed this point by scoring both SSTR3 and GPR161 localization. Please see Figure S3 for our additions.

7. Quantitation of the cells with ELMOD2 signal after ciliobrevin treatment should be provided for Fig S2. The KO control should be shown.

We have included figures for the scoring of ELMOD2 signal in ciliobrevin-treated WT cells, and we've included an image of the KO control. Please see Figure S2 for revisions.

8. Some clarification of the reproducibility of the dynamic ELMOD2 staining at rootlet structures should be provided. As presented, the observations are highly qualitative.

We have edited the text with an eye for adding more detail concerning how the experiment was done and its reproducibility. In virtually every WT cell monitored, the trends we observed and reported in ELMOD2 recruitment were consistent. Future studies aimed at parsing more details of this recruitment would likely benefit from far more quantitative datasets and related changes in the cell but were really not the focus of our studies. We have made edits to the description of these data but do not see the value of more detailed, quantitative approaches to this process to our current study.

9. A control should be provided for the overexpression of ELMOD2 in the Rootletin KO cell rescue experiment. Similarly, expression controls should be provided for the activated ARL2, ARL3, ARL6, ARF6 and ARL13B transient overexpression experiments.

It is unclear to us what exactly the reviewers meant by expression controls. We believe that our use of empty vector controls as well as the inclusion of a number of different (over)expressed constructs that had no effect on ciliogenesis rates (i.e. ARL3, ARL6, ARF6, and ARL13B) served as built-in controls verifying that overexpression/transfection alone is not sufficient to reverse ELMOD2 KO or Rootletin KO defects. We now make this point explicitly in the description of the results and discussion but see no need for additional experiments.

10. The imaging that provides the basis for the quantitations in Figure 8 should be shown.

The reviewer may have missed this, but the imaging for this quantitation is already shown in Figure S12. We chose to put these figures in the supplemental because our main figures were already large and crowded.

11. The proposed fragmentation of rootlets in the ELMOD2 knockouts is unclear- are rootlets still associated with (multiple) centriole proximal ends or are there multiple aggregates of Rootletin?

This is a great point that we now bring up in the Discussion. There are multiple aggregates of Rootletin throughout the cytoplasm that are not associated with centrosomes in ELMOD2 KO cells. In contrast, essentially all Rootletin staining in WT cells is at or surrounds centrosomes. From these data, our interpretation is that ELMOD2 is involved in recruitment to or stabilization of Rootletin at the centrosome until time for ciliation. The molecular details of potential ELMOD2-Rootletin interaction would require further study and is beyond the scope of this paper, but it provides exciting implications concerning the dynamics and the function of Rootletin (and ELMOD2) in the cell. We do see Rootletin staining at the proximal end of basal bodies, even more evident in the staining of photoreceptor cells. There are many centrosomes that have no sign of Rootletin staining, but these are invariably lacking a cilium. It might be interesting in the future to use EM to see with greater detail the relationship of Rootletin to the centrosomes in ELMOD2 KO cells.

12. The levels of Rootletin should be evaluated in ELMOD2 KO cells (this seems to be same as wild-type in Figure 4A, but this is a tangential observation there and should be tested specifically).

We repeated the Western blot directly, thus not "tangentially," with an additional two ELMOD2 KO cell lines versus WT to confirm the data shown in Figure 4A: that loss of ELMOD2 does not alter Rootletin expression. Therefore, the Rootletin defects we see in these cells come from some other avenue, most likely regulation of rootlet stability or anchoring or of rootletin polymerization. Please see Figure S15 for the new data.

13. It is unclear what is being examined in Fig 3F, particularly in the ELMOD2 KO sample. The imaging of gamma tubulin should be improved to visualise individual structures; showing fewer cells might help with this aspect. It is also unclear whether the authors are looking at individual centrioles (not quantitated) or what they score as 'centrosomes' in their separation assay. This should be clarified.

We agree that the figure showing centrosome separation was too cluttered and that having too many cells made it difficult to see the gamma tubulin staining. Once again, we were trying to show how representative the data were, but at the end of the day we caused confusion. We cleaned up the figure and chose a smaller field, and we hope that this does a better job at demonstrating our point.

14. The data for Cep44 shown in Figure S12 are not clear. There appear to be qualitative and quantitative differences in the Cep44 labelling in the various mutant cells, so this experiment should be reviewed. Quantitation of the analysis would allow a more convincing conclusion to be drawn regarding Cep44.

For the sake of clarity and because the Cep44 data aren't keys to our study of ELMOD2's functions in ciliogenesis, we decided to remove Figure S12 and the Cep44 work from this paper for both clarity and simplicity.

Minor points

15. A general point: placing the blow-ups of parts of the image within the larger image negates the purpose of having more cells in the field. Several of the images selected have redundant elements in them that contribute little and make interpretation difficult for the reader (e.g. Figs. 2A and 2D, Fig. 3F). For example, the Rootletin KO image in Fig. 4F shows a cell at right that is greatly obscured by the blow-up. The delta239 image fails to show the whole of the cell of interest. This is not ideal and the authors might help readers by simplifying their presentation.

We agree that we should make the figures cleaner/more easy to interpret. We've gone back and either moved the blow-ups mentioned or simplified the figure and now show the blow-up of the individual cilia. We were trying to include fields of cells with the hopes of highlighting that our images are representative, but we understand that at some point simplicity and clarity are important.

16. Supplemental Figures should be cited in order in the text.

We have gone back and revised the text to make sure the Supplemental Figures are appearing in the correct order.

17. The centrin antibody used should be specified as clone 20H5.

Thank you for catching this oversight- we have made the revision.

18. Figure 4 is cited instead of Figure 7 on p10, 11; this should be corrected.

And thank you also for catching this! We have gone back and revised the text.

19. Have the authors any data on what happens with expression of Rootletin 1-239? I do not suggest this as a required experiment, but it stands out as an attractive potential addition, if such data were available.

This is a really exciting question that we would love to explore one day! This is beyond the scope of this current paper as we were focusing on ELMOD2 and discovered novel functions for Rootletin along the way, but we would love to explore the functional domains of Rootletin and how these domains tie in to what factors Rootletin is interacting with. Tiansen Li in his 2002 paper showed that expression of the first 500 AA of Rootletin is globular and cannot form polymers, but this is all that's known. We would be excited to explore the N-term further as a potential auto-inhibitory domain, including perhaps comparing their 500 residue recombinant protein and our 239 residue one, perhaps exploring also the role(s) PTMs, or further dissecting the N-terminal domain as the rest of the protein is modeled with a high degree of predictability as a very cool, coiled coil structure that is enormous. Hopefully, more in the future.

RE: Manuscript #E20-10-0635R
TITLE: "Roles for ELMOD2 and Rootletin in Ciliogenesis"

Dear Dr. Kahn:

I have now received comments from the Reviewers, and as you can see both have found the manuscript much improved. Therefore, I am pleased to be able to accept your paper in principle. Before proceeding, I would like to give you the opportunity to consider introducing some of the minor textual changes suggested by Reviewer #2. Please note that I do not expect additional experiments and will not return the paper for peer review. Instead of a point by point rebuttal letter, please include the list of changes made to the revised paper in your final submission. I look forward to receiving your manuscript.
With best regards,
Fanni Gergely

Sincerely,
Fanni Gergely
Monitoring Editor
Molecular Biology of the Cell

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).

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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):

The revised manuscript from Turn and colleagues addressed a number of my concerns raised during the initial review. With regards to my comments, the authors have performed additional experiments to further show distinct roles of ELMOD2 in cell cycle versus cilia regulation, showed by immunoblotting that expression levels of centrin and Arl3 are not affected in the ELMOD2 KO cells, quantified multiciliation in cells without polyploidy (and normal centrosome number), and quantified recruitment of TTBK2 in cells with normal centrosome number. In addition, the authors have expanded the Discussion section to highlight the potential caveats with regards to ELMOD2 localization in multiple cellular compartments, and to distinguish between its roles in other cellular processes besides cilia/centrosomes. Overall, I believe the manuscript is much improved and is now suitable for publication.

Reviewer #2 (Remarks to the Author):

Turn, Kahn and colleagues have addressed the substantive issues raised in the initial review regarding cell cycle and polyploidy. The new link described here between ELMOD2, rootletin and ciliogenesis is of strong potential general interest.

However, some points remain to be clarified. I feel these should be addressed because I imagine this paper will be the basis for a good deal of future work.

1. (original review Point 9). My point was simply to show how much ELMOD2 is (over)expressed in the Rootletin KO rescue experiment and to show how much of the various other constructs (i.e., to demonstrate that they were, in fact, (over)expressed). An immunoblot was what I had envisaged, although other approaches would be fine.

2. (original review Point 10). The experiment in Fig. 8 (S13) is entirely dependent on knowing what the authors are scoring. The authors should be clearer on whether they mean centriole or centrosome in this context, given that mother/ daughter centriole localization(s) is the main issue.

Furthermore, the images shown in Fig. S13 (not S12) still do not allow the reader to determine how it was decided whether a centriole was positive or negative for CEP164, TTBK2 or CP110. This is a critical control Figure and the data presented here are notably unclear. For example, there appear to be 4 centrioles in the ELMOD2 KO cell, compared with one in the WT. I cannot determine whether these are all scored as CEP164 positive or how the authors interpret such a cell. The CP110 panels are impossible to follow (the blow-ups for cells 1 and 3 appear to be differently scaled and the merged panel doesn't show the key juxtapositions in sufficient size). This Figure should be markedly improved with a view to showing the basis for Fig. 8's scoring.

Minor points on revision
3. Size markers should be corrected in Fig. S7. Size markers should be provided for the loading controls in Figs S7, S9 and S15. Loading controls panels should be sized to match in Fig. S15.
4. Given the amount of supplemental data, I suggest Fig. S9 A-C be deleted.
5. p9 reference is made to Fig. 6A-B, which should be Fig 5; p12 refers to S13B instead of S12B.
6. p12 suggests that 'ELMOD2 acts between the Cep44 and CP110-dependent steps of the pathway' [ciliary licensing]. There is no indication of a Cep44-dependent step of ciliary licensing, so this should be corrected. p13 and the Materials and Methods also mention Cep44, which should be tidied up.

Dear Dr. Gergely,

Thank you for your “in principle” acceptance of our manuscript and thanks again to all involved in the review process for their efforts towards strengthening it. We have discussed the final edits requested and would now like to submit the final versions of the manuscript and all figures. The following is a list of changes made:

1) We added a sentence in the Methods section indicating that there are no known antibodies to ELMOD2 with the requisite sensitivity to quantify the endogenous protein by immunoblot, and later allow determination of the extent of over-expression in rescue or other experiments.

2a) We clarified both by changing our imprecise wording and by adding a full sentence to clarify, how Fig. 8 and S13 are scored; specifically using centrin staining to score centrosomes and not centrioles. We noted that the more detailed question of mother vs daughter centriole was not examined in our study.

2b) Figure S13 was modified to better highlight how scoring was performed and to document how easy it was to determine the stark differences with regard to the presence and absence of markers. This includes adjusting/normalizing the sizes of boxed areas to identify signal overlap between proteins examined.

3) Size markers were added to figures which were previously incorrectly or not so marked.

4) We chose not to delete panels from Fig. S9 as this will not significantly alter the amount of supplementary data.

5) We corrected the link to Fig. 5, not 6 and to S13.

6) We corrected the sentence on p. 12 by omitting mention of Cep44 as suggested and clarifying more precisely where we propose ELMOD2 is acting.

I hope this is clear and addresses any issue you deem appropriate and the manuscript is now ready for full acceptance.

Best,

RAK

RE: Manuscript #E20-10-0635RR
TITLE: "Roles for ELMOD2 and Rootletin in Ciliogenesis"

Dear Dr. Kahn:

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

Sincerely,
Fanni Gergely
Monitoring Editor
Molecular Biology of the Cell

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.

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