CEP55 Promotes Cilia Disassembly through Stabilizing Aurora A Kinase

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Re: JCB manuscript #202003149

Dr. Hui-Yan Li
National Center of Biomedical Analysis
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China

Dear Dr. Li,

Thank you for submitting your manuscript entitled "CEP55 Promotes Cilia Disassembly through Stabilizing Aurora A Kinase". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

The comments of reviewer #1 are especially important and must be addressed, including not only the recommend statistical tests but also all three major points. In particular, I share reviewer #1's concern about the discrepancy of cell culture data and the mouse data. I am most concerned about the data from Hek and RPE cells, and the paper would be more convincing if these experiments were performed in the mice. Of reviewer #2's comments, the data on proliferation and cilia length in different tissues in vivo is the most important to address, as these may directly relate to the phenotype of the mice. It is clear that the written english must be improved, so please pay careful attention to this issue.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

***IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data.
Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

John Wallingford, Ph.D.
Monitoring Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In their manuscript, "CEP55 promotes cilia disassembly through stabilizing Aurora A kinase," Zhang et al characterize the consequences of CEP55 loss through in vivo and in vitro analysis. They propose a model where CEP55 serves to stabilize Aurora A kinase at the base of the cilium, mediating its association with chaperonin CCT complex. The predictive model is that through Aurora A kinase, CEP55 promotes normal ciliogenesis and disassembly. They show this is not mediated by CEP55's known function in cytokinesis. Rather, a C-terminal fragment of the CEP55 protein is sufficient to protect Aurora A kinase at the centrosome. Zhang et al provide evidence that Cep55 deletion causes Meckel-Gruber syndrome-like phenotypes in mice, possibly caused by elongated cilia and a disruption of cellular mitosis. As mutations in CEP55 are linked to Meckel-Gruber syndrome, this work moves the field forward by illuminating a mechanism of how CEP55 controls cilia disassembly which may be linked to the etiology of the disease. Furthermore, the investigators provide a mouse model that could be of use to the field.

There are two major issues that need to be addressed for this work to be considered for publication. First, the manuscript requires a major editing for clarity, at the level of both logic and
syntax. At present, it requires too much work for the general reader to understand. Second, many of the statistical tests are not appropriate. Most of the data require a stronger statistical test than the student's t-test. For example, the data in Figure 2B, 2E, and 2G should be analyzed by a one-way ANOVA because there are 3 or more groups within a single variable. In others, (figure 5C and 5D) data sets have more than one grouping variable across multiple groups, therefore the data should be analyzed by a two-way ANOVA. These examples are not exhaustive and the authors should review all stats.

Assuming that those points are fixed and the significance of the data stand, the following points should be addressed:

**Major Concerns:**

1. Long and persistent cilia are a clear consequence of Cep55 deletion or knockdown and the data using serum stimulation and Aurora A rescue provide strong evidence that CEP55 normally facilitates cilia disassembly. However, the data do not really address and certainly do not support the conclusion that CEP55 negatively regulates ciliogenesis. Perhaps cells are sensitive to too much or too little CEP55 levels. Promoting disassembly would give the same phenotype as a defect in ciliogenesis. It is also possible that CEP55 functions at another locale in the cell (as IFT20 is known to do) so the cell cycle progression when Ift20 is coincidently depleted is due to that other locale's function. The authors should perform direct tests or adjust their interpretations accordingly.

2. The authors should provide the details regarding the generation of the mouse. At a descriptive level, they need to detail the "donor vector" (what does it include, ss or ds, how big?), why floxing 3-5 was chosen, how the protein and its domains align with these exons, where the antibody antigen is- editing Sfigure 1 would be an ideal place for this information. Also, how did the authors confirm that the donor did not integrate elsewhere in the genome, which would be especially concerning if at a linked locus. This information is necessary both in case anyone wanted to replicate the experiment and because it would enable us to evaluate whether this is, in fact a null allele. There may be splicing from exon 2 to ANY of the downstream exons so it is also not yet clear that this is only a loss of function. As written, there a chance that there is a small protein unrecognized by the antibody. As the experiments were done with wild type and homozygous mutant animals, it is not clear whether the hets were evaluated for phenotypes, as might occur if this created a neomorphic allele. is there a chance there could be a small protein unrecognizable by the antibody?

3. The link between the mouse data and the cell culture data make for a murky story. There does not appear to be a suggestion that more cells are ciliated in the mutant mice- only that some cilia are longer- yet the cell culture data indicate more cells are ciliated. The authors then use primary MEFs for some experiments, but jump to RPE-1 and HEK293T cells for subsequent experiments. Given the differences among cilia in different tissue types, it is not clear whether the cell culture results are in every tissue or how they relate back to the mouse. This disconnect between the in vitro and in vivo models gives the appearance that the mouse, and the physiologically relevant data, are dispensable.

**Minor Concerns:**

1. In Figure 1A, the E18.5 mice appear to display cerebellar hypoplasia, which is not listed in the paper. There is a manuscript on BioRxiv (bioRxiv 2020.04.04.024562) demonstrating a straightforward method to quantify this phenotype, which is a hallmark of Meckel-Gruber.

2. The coronal sections in figure 1A appear to be upside down.

3. Figure 2H is difficult to interpret. The figure legends are far too small. Should G0G1 and G2M be different colors? Why is there no y-axis on any of the graphs? In the x-axis "content" is misspelled.
4. Two issues with Figure 4G. 1) The highlighted region in the first column does not appear to be a cilium. Given the data in 4H, ~5% of cells in this condition have cilia. Is there a better example? 2) Why is the red channel background so high in conditions that are not expressing mCherry? Why is it so low in conditions that do express mCherry?

5. The authors should be more clear how the allele they generate relates to the the C>T256 mutation. According to Bondeson et al 2017, this nonsense mutation causes a premature stop-codon and a truncated protein. In this manuscript, the authors show that a plasmid with the C>T256 mutation creates what appears to be a single truncated protein in figure 4F. Do these data agree with the interpretation from Bondeson et al?

6. It is difficult to understand the analysis of data in figure 5C. Are the experimental groups being compared to their respective time-point controls in the wildtype condition? These details should be added to the figure legend.

7. The mechanism of stabilizing AuroraA implies that this mechanism works through HDAC6, which is examined in several figures, but never discussed. This should be included in the discussion.

8. The end tangent on cancer comes out of left field and doesn't add to the manuscript.

Reviewer #2 (Comments to the Authors (Required)):

Zhang et al evaluates the role of the centrosomal and mid-body protein Cep55 in cilia function, noting that Cep55 is mutated in MKS. They evaluate the phenotype of Cep55-/- mice and note that these exhibit defects that at least partially recapitulate those of human MKS. Interestingly however, unlike other mutants linked to MKS and other ciliopathies, loss of Cep55 leads to longer cilia and more cilia in MEFs. The authors go on to identify a mechanism by which Cep55 acts through AurA kinase to regulate cilium assembly and length. This is a generally well performed study that gets at the important question of how cilia are regulated. It combines in vivo and cell culture assays to address this question. I generally think this paper should be published, however, there are some additional experiments and considerations that I think would improve the work.

Major Questions/Concerns:

The fact that the authors identify a ciliopathy-associated protein that seems to negatively regulate cilia is interesting and fairly novel. A more thorough characterization of the actual cilia phenotype in the Cep55-/- mice and cells is therefore important. Are there more and longer cilia in vivo as well as in the MEFs? This is looked at in the kidneys and choroid plexus, but might be useful to quantify primary cilia number in the limbs or facial mesenchyme. It would just be interesting to know if cilia numbers are increased in vivo given the links to AurA.

It would be good to investigate whether trafficking (IFT protein localization) or signaling (localization of Hh pathway or other signaling components related to the phenotype) in these cilia is abnormal and/or to characterize the cilia structure by TEM.

The authors note a cell cycle defect in the cultured cells. Are there any in vivo changes in cell cycle-changes in Ki67, phospho-histone3, etc in tissues affected in the mutants, such as the brain? Additional characterization of Cep55 protein localization would also be informative. It is reported to be present on interphase centrosomes. But at what specific stages of the cell cycle is Cep55 found at the centrosome? Does it co-localize with phospho-AurA kinase at the centrosome? Is the MKS-associated mutation impaired in its localization to the centrosome? This is shown in passing in Figure 4, but should be quantified.

Do any or all of the CCT proteins localize to the centrosome? This should be investigated with either antibodies (if available) or tagged protein of some sort. Does localization change in the absence of Cep55?
From the Western in Figure 6C, it was unclear to me whether knockdown of Cep55 is associated with reduced CCT protein expression for some or all CCTs examined. Are the authors arguing this is the case? This was also unclear in the text. If not, what do they propose to be the connection between CEP55 and CCTs? Regardless, quantification of the westerns to establish that protein levels are changed in Fig 6C is important.

Additional points:

On page 10, the authors write "Consistently, this C256T mutant failed to bind to Aurora A (Fig. 4 F) and rescues increased ciliation induced by CEP55 depletion (Fig. 4, G and H), suggesting that the C256T mutant loses the inhibitory activity on ciliogenesis." I think they meant to say "failed to rescue increased ciliation."
Point-to-point responses to the reviewers’ concerns.

**Reviewer #1**

_In their manuscript, “CEP55 promotes cilia disassembly through stabilizing Aurora A kinase,” Zhang et al characterize the consequences of CEP55 loss through in vivo and in vitro analysis. They propose a model where CEP55 serves to stabilize Aurora A kinase at the base of the cilium, mediating its association with chaperonin CCT complex. The predictive model is that through Aurora A kinase, CEP55 promotes normal ciliogenesis and disassembly. They show this is not mediated by CEP55’s known function in cytokinesis. Rather, a C-terminal fragment of the CEP55 protein is sufficient to protect Aurora A kinase at the centrosome. Zhang et al provide evidence that Cep55 deletion causes Meckel-Gruber syndrome-like phenotypes in mice, possibly caused by elongated cilia and a disruption of cellular mitosis. As mutations in CEP55 are linked to Meckel-Gruber syndrome, this work moves the field forward by illuminating a mechanism of how CEP55 controls cilium disassembly which may be linked to the etiology of the disease. Furthermore, the investigators provide a mouse model that could be of use to the field._

There are two major issues that need to be addressed for this work to be considered for publication. First, the manuscript requires a major editing for clarity, at the level of both logic and syntax. At present, it requires too much work for the general reader to understand. Second, many of the statistical tests are not appropriate. Most of the data require a stronger statistical test than the student's t-test. For example, the data in Figure 2B, 2E, and 2G should be analyzed by a one-way ANOVA because there are 3 or more groups within a single variable. In others, (figure 5C and 5D) data sets have more than one grouping variable across multiple groups, therefore the data should be analyzed by a two-way ANOVA. These examples are not exhaustive and the authors should review all stats.

**Response:** We are grateful to the reviewer for the cogent summary of the major contributions of our study and the overall positive assessment. Many thanks for raising these two important issues. Following these suggestions, we have extensively rewritten our manuscript at the level of both logic and syntax, to make it easier to read and understand. We have highlighted all changes in our revised manuscript text file.

In addition, we have carefully checked every graph and applied appropriate statistical analysis methods. The data in revised Fig. 2F, 2I, 3B, 3E, 3G, 4F, 4K, 5G, 5J and 6F are analyzed by one-way ANOVA, and the data in revised Fig. 2M, 5C, 5D and 6D are analyzed by two-way ANOVA. Meanwhile, we have stated the name of the statistical test in the associated legends, and modified the text of the Methods/Statistical analysis section in our revised manuscript.

**Major Concerns:**

1. Long and persistent cilia are a clear consequence of Cep55 deletion or knockdown and the data using serum stimulation and Aurora A rescue provide strong evidence that CEP55 normally facilitates cilia disassembly.
However, the data do not really address and certainly do not support the conclusion that CEP55 negatively regulates ciliogenesis. Perhaps cells are sensitive to too much or too little CEP55 levels. Promoting disassembly would give the same phenotype as a defect in ciliogenesis. It is also possible that CEP55 functions at another locale in the cell (as IFT20 is known to do) so the cell cycle progression when Ift20 is coincidently depleted is due to that other locale's function. The authors should perform direct tests or adjust their interpretations accordingly.

Response: We appreciate the reviewer for pointing out this issue and giving us valuable suggestions. As the reviewer commented that “Aurora A rescue provide strong evidence that CEP55 normally facilitates cilia disassembly”, our data showed that CEP55 depletion promotes cilia formation more likely due to disrupting cilia disassembly, rather than facilitating cilia assembly. Therefore, in the revised manuscript, we have adjusted our interpretations and revised all of “negatively regulate ciliogenesis” into “negatively regulate cilia formation” (For example, the title of Fig.3 in revised manuscript is “CEP55 negatively regulates cilia formation in cultured cells”).

2. The authors should provide the details regarding the generation of the mouse. At a descriptive level, they need to detail the “donor vector” (what does it include, ss or ds, how big?), why floxing 3-5 was chosen, how the protein and its domains align with these exons, where the antibody antigen is editing $figure 1 would be an ideal place for this information. Also, how did the authors confirm that the donor did not integrate elsewhere in the genome, which would be especially concerning if at a linked locus. This information is necessary both in case anyone wanted to replicate the experiment and because it would enable us to evaluate whether this is, in fact a null allele. There may be splicing from exon 2 to ANY of the downstream exons so it is also not yet clear that this is only a loss of function. As written, there a chance that there is a small protein unrecognized by the antibody. As the experiments were done with wild type and homozygous mutant animals, it is not clear whether the hets were evaluated for phenotypes, as might occur if this created a neomorphic allele. is there a chance there could be a small protein unrecognizable by the antibody?

Response: We apologized for leaving out the detailed descriptions about the generation of the mouse. We have added the detailed information in our revised manuscript and now these descriptions are listed as follows:

First, both of these two donor vectors are 1.9kbp dsDNA, containing homologous arms, loxp sites and restriction sites. We have added these descriptions to the Materials and Methods/Mice section in the revised manuscript (Page 19, Line 6).

Second, the reason why we chose floxing exon3 to 5 is that: the start codon of Cep55 protein locates at exon2, so we inserted 5’loxp site at the downstream of exon2, namely the upstream of exon3. On the other hand, inserting 3’loxp site at the downstream of exon5 caused a deletion of exon3-5, which further result in a frameshift. Thus, floxing exon3-5 is the optimal strategy for knocking out Cep55. Besides, following the reviewer’s suggestion, we have added the schematic representation of Cep55 protein containing its domain information, and aligned with all the exons in revised Fig. S1A.
Third, the antigen of anti-Cep55 antibody used in our original manuscript is a fragment containing C-terminal residues around the Val459 of human CEP55 protein. To exclude the restriction of this antibody, we also confirmed the efficiency of knocking out Cep55 by using another antibody (Abnova, H00055165-A01) against a full-length recombinant CEP55 protein (in revised Fig. S1D). These results indicated that a small unrecognizable protein seems unlikely to be remained in Cep55−/− mice.

To exclude the possibility that the donors integrate elsewhere in the genome, we have performed a southern blot assay in the modified mice. The result showed that the donors did not integrate elsewhere in the genome. The data showed in the below.

Finally, as the reviewer mentioned, we evaluated the heterozygous mice as well. Compared to wild-type mice, the heterozygote did not exhibit any obvious abnormalities in histological morphology or cilia formation.

3. The link between the mouse data and the cell culture data make for a murky story. There does not appear to be a suggestion that more cells are ciliated in the mutant mice- only that some cilia are longer- yet the cell culture data indicate more cells are ciliated. The authors then use primary MEFs for some experiments, but jump to RPE-1 and HEK293T cells for subsequent experiments. Given the differences among cilia in different tissue types, it is not clear whether the cell culture results are in every tissue or how they relate back to the mouse. This disconnect between the in vitro and in vivo models gives the appearance that the mouse, and the physiologically relevant data, are dispensable.

Response: We thank the reviewer for raising this important issue and apologized for our unclear descriptions in our original manuscript. Our original data showed that CEP55-depleted RPE-1 cells obviously increased both the percentage (+Serum) and length (−Serum) of cilia. However, in Cep55-deficient mice, only that some cilia are longer, no more cells are ciliated. Thus, we observed the increased length of cilia in both cultured cells and mice when Cep55 is deficient. Intriguingly, why there are no more cells are ciliated in Cep55-deficient mice, we proposed that the ability to form a cilium for individual cells is strictly determined by their lineage during embryonic development, even though they share the adjacent location (Bangs et al., Nat Cell Biol, 2015). This lineage-dependent mechanism determines that there are always two different status cells in tissues, including the ciliated cells and non-ciliated cells. When disassembly is disrupted, the ciliated cells could only display a longer cilium, however, the non-ciliated cells could hardly be ciliated since cilia assembly machine most likely cannot work here. Therefore, Cep55-null mice only show longer cilia in some tissues, while the percentage of ciliated cells seems no change. Meanwhile, we took this issue seriously and consulted with several cilia experts, Dr. Jeremy Reiter (University of California, San Francisco), Dr. Brian David Dynlacht (New York University School of Medicine) and Dr. Michel R Leroux (Simon Fraser University). They also coincide with our opinions and believe that this phenomenon is very
frequent and reasonable. Considering this issue is very important, we have added this into the discussion section in our revised manuscript (Page 17, Line 8).

As for using different cell lines to perform our experiments, we summarized as follows. In RPE-1 cells and primary MEFs, our original data revealed that Cep55 deficiency increased the percentage and length of cilia through suppressing cilia disassembly, and led to a reduction of Aurora A protein level during cilia disassembly. Additionally, in RPE-1 cells and HEK293 cells, we showed that CEP55 could specifically bind to Aurora A in our original manuscript. Furthermore, we confirmed the endogenous interaction between Cep55 and Aurora A in primary MEFs (in revised Fig. 4C). Taken together, we demonstrated that CEP55 promotes cilia disassembly by interacting and stabilizing Aurora A kinase in above three cells, suggesting that the role of CEP55 in controlling cilia disassembly is well-conserved.

Minor Concerns:

1. In Figure 1A, the E18.5 mice appear to display cerebellar hypoplasia, which is not listed in the paper. There is a manuscript on BioRxiv (bioRxiv 2020.04.04.024562) demonstrating a straightforward method to quantify this phenotype, which is a hallmark of Meckel-Gruber.

Response: Many thanks for the constructive suggestions. This is very useful for us and will strengthen our findings. By using the smart analysis method mentioned in that elegant work, we quantified the size of the cerebellum and found that Cep55−/− mice displayed an extremely smaller cerebellum than wild-type (in revised Fig. 1F and 1G). Thus, this result further strengthened the relevance between Cep55 deletion and MKS. We have cited the related paper in our revised manuscript (Page 6, Line 8).

2. The coronal sections in figure 1A appear to be upside down.

Response: We are grateful to this kind reminder. In our revised version, we have modified this figure following the reviewer’s suggestion.

3. Figure 2H is difficult to interpret. The figure legends are far too small. Should G0G1 and G2M be different colors? Why is there no y-axis on any of the graphs? In the x-axis "content" is misspelled.

Response: Many thanks to the reviewer’s keen observation and constructive suggestion. In our revised manuscript, we have labeled the percentages of cells in different cell cycle stages in the graph directly (in revised Fig. 2H). In addition, we have added the number to the y-axis and corrected our misspelling in the x-axis as well in this figure.

4. Two issues with Figure 4G. 1) The highlighted region in the first column does not appear to be a cilium. Given the data in 4H, ~5% of cells in this condition have cilia. Is there a better example? 2) Why is the red channel background so high in conditions that are not expressing mCherry? Why is it so low in conditions that do express mCherry?
Response: Thanks for raising these important issues. 1) In our original Fig.4G, the highlighted region of representative image from the control group showed that a cell has no cilia, which represents the status of most cells in the control group. 2) We apologized for the unclear description on the minus signs in our original Fig.4G legends. The control cells were transfected with mCherry-vector, so the red positive signal could be observed in their cytoplasm. Unlike the mCherry-vector, mCherry-Cep55 localized at the centrosome rather than in the cytoplasm in mCherry-CEP55-transfected cells, which results in a lower red signal in the cytoplasm. We have modified our description on the label in our revised Fig.4G legends.

5. The authors should be more clear how the allele they generate relates to the the C>T256 mutation. According to Bondeson et al 2017, this nonsense mutation causes a premature stop-codon and a truncated protein. In this manuscript, the authors show that a plasmid with the C>T256 mutation creates what appears to be a single truncated protein in figure 4F. Do these data agree with the interpretation from Bondeson et al?

Response: We appreciate the reviewer for pointing out this issue. The nonsense mutation c.256C>T in CEP55 identified by Bondeson et al. causes the Arg (CGA) turn to a stop codon (TGA) at position 86, which resulted in a truncated protein (1-85 aa). In our original manuscript, we constructed the mCherry-CEP55 C256T mutant plasmid and verified it by sequencing. This C256T mutant plasmid only encoded the N-terminal truncation of CEP55 (1-85 aa), which completely mimics the truncated protein reported by Bondeson et al.

6. It is difficult to understand the analysis of data in figure 5C. Are the experimental groups being compared to their respective time-point controls in the wildtype condition? These details should be added to the figure legend.

Response: We apologized for the lack of detailed description of the statistical analysis in Fig. 5C. We have added the detailed information to the legends of Fig. 5C in the revised manuscript (Page 44, Line 6).

7. The mechanism of stabilizing AuroraA implies that this mechanism works through HDAC6, which is examined in several figures, but never discussed. This should be included in the discussion.

Response: We are grateful to the reviewer’s suggestion. Our original data showed that CEP55 could not bind to HDAC6, and depletion of CEP55 did not affect the stability of HDAC6. Mechanistically, we demonstrated that CEP55 stabilizes the protein level of Aurora A, through mediating the interaction between Aurora A and chaperonin CCT complex. It is suggested that CEP55 stabilized Aurora A mainly though CCT complex, which probably functions upstream of HDAC6. As the reviewer kindly suggested, we have added these related descriptions into our results (Page 10, Line 19) and discussion (Page 16, Line 15).

8. The end tangent on cancer comes out of left field and doesn't add to the manuscript.

Response: Thanks for the constructive suggestion. We have removed these descriptions as suggested.
Reviewer #2

Zhang et al evaluates the role of the centrosomal and mid-body protein Cep55 in cilia function, noting that Cep55 is mutated in MKS. They evaluate the phenotype of Cep55−/− mice and note that these exhibit defects that at least partially recapitulate those of human MKS. Interestingly however, unlike other mutants linked to MKS and other ciliopathies, loss of Cep55 leads to longer cilia and more cilia in MEFs. The authors go on to identify a mechanism by which Cep55 acts through AurA kinase to regulate cilium assembly and length. This is a generally well performed study that gets at the important question of how cilia are regulated. It combines in vivo and cell culture assays to address this question. I generally think this paper should be published, however, there are some additional experiments and considerations that I think would improve the work.

Major Concerns:

1. The fact that the authors identify a ciliopathy-associated protein that seems to negatively regulate cilia is interesting and fairly novel. A more thorough characterization of the actual cilia phenotype in the Cep55−/− mice and cells is therefore important. Are there more and longer cilia in vivo as well as in the MEFs? This is looked at in the kidneys and choroid plexus, but might be useful to quantify primary cilia number in the limbs or facial mesenchyme. It would just be interesting to know if cilia numbers are increased in vivo given the links to Aurora A.

Response: We sincerely thank the reviewer for his/her interest in our study and raising this important issue. We have performed histological analysis and detected the ciliogenesis in Cep55−/− limbs and facial mesenchyme. Data showed that no remarkable histological abnormalities or aberrant cilia formation were observed in these tissues of Cep55−/− mice (in revised Fig. S1E-S1H), which is consistent with the phenotypes in CEP55 mutation human fetuses, none of whom had polydactyly or facial malformation as previously reported (Bondeson et al., Clinical genetics, 2017).

Given the multiple roles of cilia in development and physiology, it is accepted that mutations of different ciliary genes can frequently give rise to partially overlapping or totally distinct phenotypes, which are classified into diversified ciliopathies (Karmous-Benailly et al., Am J Hum Genet, 2005; Valente et al., Eur J Med Genet, 2008). In addition, phenotypes of ciliopathies are often exhibited at single or multiple organ, since cillum is a complex organelle with variable structure-based functions in different tissue contexts (Salonen et al., J Med Genet, 1998; Marshall et al., The Journal of Cell Biology, 2008).

Besides, according to the reviewer’s advice, we separated E9.5 neural tubes of wild-type and Cep55−/− embryos, then lysed and blotted with Aurora A antibody. Our data showed that total Aurora A levels decreased in Cep55−/− mice, compared with wild-type or heterozygous mice (in revised Fig. S4C). Thus, we speculate that Cep55-deficient mice induced abnormal cilia formation likely through destabilizing Aurora A protein.
2. It would be good to investigate whether trafficking (IFT protein localization) or signaling (localization of Hh pathway or other signaling components related to the phenotype) in these cilia is abnormal and/or to characterize the cilia structure by TEM.

Response: Many thanks for the constructive suggestions. We have investigated whether the Hedgehog signaling is affected in Cep55−/− mice in our revised manuscript. In response to SAG (SMO Agonist) treatment, SMO localized to cilia in wild-type MEFs but not in Cep55−/− MEFs (in revised Fig. 2L and 2M). These results indicated that Cep55-mediated proper ciliogenesis is critical for Hedgehog signal transduction.

3. The authors note a cell cycle defect in the cultured cells. Are there any in vivo changes in cell cycle- ie changes in Ki67, phospho-histone3, etc in tissues affected in the mutants, such as the brain?

Response: We are grateful to the reviewer’s suggestions and have performed experiments accordingly. To confirm the effect of Cep55 on cell proliferation in vivo, we have examined Ki67 expression in the cerebral cortex and found that Cep55−/− mice had a 55% reduction in cell proliferation compared to wild-type mice (in revised Fig. 2J and 2H). Further immunostaining of pH3 in cortical sections revealed little decrease in mitosis cells in Cep55−/− cortices (in revised Fig. S2A and S2B). These results supported that Cep55 deficiency mainly arrested cell at the G0/G1 phase but not mitosis, consistent with our original data in Cep55−/− MEFs. We have shown these results in the revised manuscript.

4. Additional characterization of Cep55 protein localization would also be informative. It is reported to be present on interphase centrosomes. But at what specific stages of the cell cycle is Cep55 found at the centrosome? Does it co-localize with phospho-AurA kinase at the centrosome? Is the MKS-associated mutation impaired in its localization to the centrosome? This is shown in passing in Figure 4, but should be quantified.

Response: We appreciate the reviewer for pointing out this issue. Following the reviewer’s suggestion, we have verified the endogenous localization of CEP55 in RPE cells in our revised manuscript. Microscopy analysis revealed that CEP55 localized to the centrosome in G0/G1 phase, and to one of the two centrioles in S/G2 phase, then moved to midbody during cytokinesis (in revised Fig. S3F–S3H). These results are consistent with previous studies (Fabbro et al., Developmental cell, 2005; Martinez-Garay et al., Genomics, 2006).

Additionally, we have tested the localization of phospho-AurA and CEP55 during cilia disassembly. After serum stimulation, the phospho-AurA (T288) and CEP55 are colocalized at the centrosome (in revised Fig. S4C).

To identify the localization of the MKS-associated mutation in CEP55, RPE-1 cells were transfected with mCherry-CEP55 C256T plasmid and stained with the centrosomal marker γ-tubulin. Our data showed that unlike CEP55 wild type, CEP55 C256T mutant could not localize to the centrosome (in revised Fig. 4H). We have added all these above results in the revised manuscript.
5. Do any or all of the CCT proteins localize to the centrosome? This should be investigated with either antibodies (if available) or tagged protein of some sort. Does localization change in the absence of Cep55?

Response: Thanks for the critical and constructive suggestion. Previous study described that most CCT proteins localize to the centrosome, except for CCT2 (Seo et al., PNAS, 2010). We have confirmed their cellular localizations with immunofluorescence microscopy (in revised Fig. S6C–S6F). Our results showed that knockdown of CEP55 did not affect the localization of CCT proteins (in revised Fig. S6C–S6F). It is possible that CEP55 functions as a chaperonin-like protein interacting with CCT complex to stabilize Aurora A. We have shown our results in the revised manuscript.

6. From the Western in Figure 6C, it was unclear to me whether knockdown of Cep55 is associated with reduced CCT protein expression for some or all CCTs examined. Are the authors arguing this is the case? This was also unclear in the text. If not, what do they propose to be the connection between CEP55 and CCTs? Regardless, quantification of the westerns to establish that protein levels are changed in Fig 6C is important.

Response: Many thanks for this suggestion. We have quantified the westerns in original Fig.6C with Image J software. The data showed that the levels of CCT proteins have no remarkable reduction in CEP55-depleted RPE-1 cells (see revised Fig. 6C). Together with our immunofluorescent data in revised Fig. S6C–S6F, we believe that CEP55 deficiency hardly affects the stability of CCT proteins. According to the above results and our original data, we proposed a model for CEP55-Aurora A-CCT complex: CEP55 binds to Aurora A, and then facilitates the interaction between Aurora A and some CCT subunits to stabilize Aurora A protein level. When the chaperonin-like protein CEP55 was deficient in binding Aurora A, the Aurora A-CCT complex tends to disassociate, resulting in the instability of Aurora A. However, how CCT complex contributed to stabilizing the protein level of Aurora A remains to be addressed in our future study.

Additional points:

On page 10, the authors write "Consistently, this C256T mutant failed to bind to Aurora A (Fig. 4 F) and rescues increased ciliation induced by CEP55 depletion (Fig. 4, G and H), suggesting that the C256T mutant loses the inhibitory activity on ciliogenesis." I think they meant to say "failed to rescue increased ciliation."

Response: We thank the reviewer for pointing out this important issue and giving us a chance to correct our negligence. We have corrected our statement in our revised manuscript as “Consistently, this C256T mutant failed to bind to Aurora A (Fig. 4 I) and cannot rescue increased ciliation induced by CEP55 depletion (Fig. 4, J and K), suggesting that the C256T mutant loses the inhibitory activity on ciliogenesis”
October 19, 2020

RE: JCB Manuscript #202003149R

Dr. Hui-Yan Li
National Center of Biomedical Analysis
27, taiping road
Beijing 100850
China

Dear Dr. Li:

Thank you for submitting your revised manuscript entitled "CEP55 Promotes Cilia Disassembly through Stabilizing Aurora A Kinase". The paper has now been seen again by the original reviewers who both recommend acceptance. Therefore, we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

**As you will see, both reviewers have raised some minor points that will need to be addressed prior to official acceptance of the paper. These changes should not require any new experiments and can likely be addressed by changes to the text and re-analyses of the statistical methods. Please be sure to include a point-by-point rebuttal to these remaining concerns along with your final revision.**

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends. You are well below this limit at this time but please bear it in mind when revising.

2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test.
(for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, since you
used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined
whether the data was normally distributed before selecting that test. In the stats section of the
methods, you do indicate that you tested for normal distribution of the data but you do not indicate
what test you used to determine normality - please provide that information in the methods
section.

4) Materials and methods: Should be comprehensive and not simply reference a previous
publication for details on how an experiment was performed. Please provide full descriptions (at
least in brief) in the text for readers who may not have access to referenced manuscripts. The text
should not refer to methods "...as previously described."

5) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the
materials and methods. You must also indicate in the methods the source, species, and catalog
numbers (where appropriate) for all of your antibodies.

6) Microscope image acquisition: The following information must be provided about the acquisition
and processing of images:
   a. Make and model of microscope
   b. Type, magnification, and numerical aperture of the objective lenses
   c. Temperature
   d. Imaging medium
   e. Fluorochromes
   f. Camera make and model
   g. Acquisition software
   h. Any software used for image processing subsequent to data acquisition. Please include details
      and types of operations involved (e.g., type of deconvolution, 3D reconstructions, surface or volume
      rendering, gamma adjustments, etc.).

7) References: There is no limit to the number of references cited in a manuscript. References
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8) Supplemental materials: There are normally strict limits on the allowable amount of supplemental
data. Articles/Tools may usually have up to 5 supplemental figures. At the moment, you have 6
supplementary figures. In this case, we will be able to give you the extra space but try not to add to
the current total.
   Please also note that tables, like figures, should be provided as individual, editable files. A summary
   of all supplemental material should appear at the end of the Materials and methods section.

9) eTOC summary: A ~40-50 word summary that describes the context and significance of the
findings for a general readership should be included on the title page. The statement should be
written in the present tense and refer to the work in the third person. It should begin with "First
author name(s) et al..." to match our preferred style.

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

John Wallingford, PhD
Monitoring Editor
Journal of Cell Biology
Reviewer #1 (Comments to the Authors (Required)):

The authors have done a fantastic job addressing the majority of comments and have greatly improved their manuscript. There are three minor comments that I believe need to be addressed before publication.
1) There is still a question of how the data for cilia formation are being interpreted. This is best illustrated in Figure 5. The authors show that CEP55 null cells (5D) and siCEP55 treated cells (5C) still form cilia at normal rates under serum starved conditions. In the presence of serum, normal cilia degenerate but in mutant cell lines cilia are persistent. These data show that CEP55 is dispensable for cilia formation, but it is critical in cilia disassembly.
So, the wording "negatively regulating ciliogenesis" or "negatively regulating cilia formation" is not supported by the presented data, because ciliogenesis/cilia formation occurs normally. The wording needed to be changed to accurately reflect the results.
2) The majority of the statistical tests are now appropriate. However, there are some cases where the wrong test was used. In Panels 2F, 5J and 6F the authors should have analyzed their data with a two-way ANOVA rather than a one-way ANOVA. While I don't think this changes the result, it is critical that it be corrected.
3) In Figure 2 the authors conclude that Hedgehog signaling is restricted, but their data do not support this conclusion. Smo enrichment in cilia is a hallmark of pathway activation, but ciliary Smo is not a readout of pathway activation. The language here and in the results section should be amended. The phenotype and observed decrease of Smo in cilia demonstrate abnormal Smo enrichment. While the results may be consistent with a decrease in Shh signaling, future experiments will have to examine Hedgehog pathway activity.
Page 17, line 14-15 grammar.

Reviewer #2 (Comments to the Authors (Required)):

This manuscript is greatly improved since the prior version, and the authors have addressed all of my concerns regarding data. My one minor point is the text still contains grammatical errors and might benefit from copy editing. In particular the new Discussion section added about cilia in specific lineages (page 17) was somewhat unclear. Authors should rewrite or perhaps consider creating a model figure to summarize their point. The data are strong and support the conclusions of the paper however, and the paper has novel findings that advance the field. I don't think it requires re-review- just some copy editing.
Point-to-point responses to the reviewers’ concerns.

Reviewer #1

The authors have done a fantastic job addressing the majority of comments and have greatly improved their manuscript. There are three minor comments that I believe need to be addressed before publication.

1) There is still a question of how the data for cilia formation are being interpreted. This is best illustrated in Figure 5. The authors show that CEP55 null cells (5D) and siCEP55 treated cells (5C) still form cilia at normal rates under serum starved conditions. In the presence of serum, normal cilia degenerate but in mutant cell lines cilia are persistent. These data show that CEP55 is dispensable for cilia formation, but it is critical in cilia disassembly.

So, the wording "negatively regulating ciliogenesis" or "negatively regulating cilia formation" is not supported by the presented data, because ciliogenesis/cilia formation occurs normally. The wording needed to be changed to accurately reflect the results.

Response: We are grateful to the reviewer’s encouraging comments on our manuscript. We agree with the reviewer on this point, and have modified our statements and interpretations to accurately describe the results. (For example, the title of Fig.3 in revised manuscript is “Depletion of CEP55 increased the percentage and length of primary cilia in cultured cells”)

2) The majority of the statistical tests are now appropriate. However, there are some cases where the wrong test was used. In Panels 2F, 5J and 6F the authors should have analyzed their data with a two-way ANOVA rather than a one-way ANOVA. While I don't think this changes the result, it is critical that it be corrected.

Response: We appreciate the reviewer for pointing out this issue. Following the suggestion, we have adjusted the statistical tests to two-way ANOVA in revised Fig. 2F, 5J and 6F, and modified the associated legends in our revised version.

3) In Figure 2 the authors conclude that Hedgehog signaling is restricted, but their data do not support this conclusion. Smo enrichment in cilia is a hallmark of pathway activation, but ciliary Smo is not a readout of pathway activation. The language here and in the results section should be amended. The phenotype and observed decrease of Smo in cilia demonstrate abnormal Smo enrichment. While the results may be consistent with a decrease in Shh signaling, future experiments will have to examine Hedgehog pathway activity.

Response: Many thanks for the constructive suggestions and providing directions for our further research. We have revised the statement to “Together, these results indicate that Cep55 deficiency resulted in decreased Smo enrichment, which possibly led to defect in Hh signal transduction.” in Page 9 line 11-13.

Page 17, line 14-15 grammar.

Response: We thank the reviewer for raising this important issue. We have modified the
Reviewer #2

This manuscript is greatly improved since the prior version, and the authors have addressed all of my concerns regarding data. My one minor point is that the text still contains grammatical errors and might benefit from copy editing. In particular, the new Discussion section added about cilia in specific lineages (page 17) was somewhat unclear. Authors should rewrite or perhaps consider creating a model figure to summarize their point. The data are strong and support the conclusions of the paper however, and the paper has novel findings that advance the field. I don't think it requires re-review—just some copy editing.

Response: We are grateful to the reviewer for the positive assessment. Following his/her suggestions, we have rewritten the newly added paragraph to describe our model more clear. “Note that the ability to form a cilium for individual cells is strictly determined by their lineage during embryonic development (Bangs et al. 2015), even though they share the adjacent location in the same tissue. Thus, the cells are overall in two different status, ciliated or non-ciliated. When disassembly components are disrupted, the cilium on ciliated cells could display longer. However, non-ciliated cells still could not form a cilium due to the shortage of assembly machine. Therefore, Cep55-null mice only show increased ciliary length in tissues, but unaltered percentage of ciliated cells.”