JCB

LUBAC regulates ciliogenesis by promoting CP110 removal from the mother centriole

Xiao-Lin Shen, Jinfeng Yuan, Xuan-He Qin, Guang-ping Song, Huaibin Hu, Haiqing Tu, Zengqing Song, Pei-Yao Li, Yu-Ling Xu, Sen Li, Xiao-Xiao Jian, Jia-Ning Li, Chun-Yu He, Xi-Ping Yu, Li-Yun Liang, Min Wu, Qiu-Ying Han, Kai Wang, Ailing Li, Tao Zhou, Yucheng Zhang, Na Wang, and Hui-Yan Li

Corresponding Author(s): Hui-Yan Li, National Center of Biomedical Analysis and Na Wang, National Center of Biomedical Analysis

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July 20, 2021

Re: JCB manuscript #202105092

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

Dear Dr. Li,

Thank you for submitting your manuscript entitled "LUBAC promotes ciliogenesis by regulating CP110 removal from the mother centriole.". The manuscript was assessed by expert reviewers, whose comments are appended to this letter.

You will see that all reviewers are enthusiastic about your study but raise a number of points that we believe are essential to address in order to further support your conclusions. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

1) Reviewers #1&3 ask to show the interphase localization of the LUBAC complex, which we would expect to be at the mother centriole prior to cilium assembly in cycling G1 cells and lost after the cilium emerges in G0. Likewise, linear Ubiquitin chains should be detectable at the mother centriole prior to cilium assembly.

2) Reviewer #2 requests to investigate the functional hierarchy between CP110 and LUBAC by testing whether depletion of CP110 rescue ciliogenesis in LUBAC-depleted cells. This Reviewer also asks to clarify the mechanism by which PRPF8 removes CP110-linearUb from the mother centriole, suggesting possible experiments but you should feel free to design experiments as needed in order to provide additional insights into the molecular mechanism of this process.

3) Reviewer #3 notes that the HOIP mutant used as catalytically inactive is not appropriate and states this experiment must be repeated with a HOIP C885S mutant. This mutant should also be added as a control to co-IP experiments in Fig. 5B.

The remaining requests from Reviewers; rescues in zebrafish, in vitro ubiquitylation assays, evidence for an endogenous complex, and studies with more targeted PRPF8 mutations are all interesting but we do not believe these are essential for revision. Reviewers also raise various minor points that require text and figure revisions which must be addressed. Of particular importance here are comments from Reviewer #2 regarding lack of discussion of prior papers and insufficient descriptions of methods and materials.

While revising your paper please note that per JCB policy the materials and methods should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. The text should not refer to methods or constructs as "previously described" or "already exist in our lab." For all cell lines, vectors, constructs/cDNAs, etc: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic

features, even if described in other published work or gifted to you by other investigators, and include references where appropriate. You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary.

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, https://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 measures to 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. If you are faced with such restrictions we recommend that you reach out to the editors 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,

Maxence Nachury, PhD

Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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

In this report Shen et al, provide extensive evidence supporting a role for the linear ubiquitin chain assembly complex (LUBAC) in the removal of the CP110-CEP97 from the mother centriole during ciliogenesis. They further propose that the splicing factor PRPF8 acts as a receptor for linear ubiquitinated CP110 which promote its removal thereby driving ciliogenesis.

This very interesting report, however, is not yet ready for the readership of the Journal of Cell Biology. Several important pieces of data are lacking.

1) The authors stated that the linear ubiquitin chain assembly complex (LUBAC) was localized to the centrosomes in interphase cells. No immunofluorescence data or a reference was provided to support this statement. When in interphase is LUBAC localized to the centrosome?

2) The authors propose that LUBAC and CP110 form a robust complex, but this was shown by overexpression in 293 cells. Please provide evidence of the endogenous complex in RPE1 cells.

3) LUBAC ubiquitylation of CP110 would be more convincing if the authors did this assay in vitro using purified components.

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

In this manuscript, Shen et al. examine the regulation of CP110, a protein that caps the mother centriole and acts as a key negative regulator of cilium assembly. Shen et al. report that the LUBAC ubiquitin ligase acts with PRPF8 to promote ciliogenesis via ubiquitination of CP110 and removal of CP110 from the mother centriole. Despite some issues and caveats noted below, overall this study provides convincing evidence that LUBAC assembles linear ubiquitin chains on CP110, that ubiquitinated CP110 is recognized by PRPF8, and that LUBAC and PRPF8 are necessary for CP110 removal and cilium assembly. The characterization and application of the CP110 allele that does not bind LUBAC or get removed from the mother centriole is particularly valuable. While prior studies have also examined CP110 regulation and identified CP110-modifying ubiquitin ligases (such as Neurl4 and EDD1-DDB1-VPRBP), the molecular pathways responsible for CP110 removal remain incompletely characterized, and the cellular functions of linear ubiquitin chains are also not fully understood.
The manuscript would be improved through further examination of the functional relationship between LUBAC and PRPF8, through evaluation of whether CP110 RNAi bypasses the roles of LUBAC/PRPF8 in ciliogenesis, and through better integration of the present findings with prior

reports on regulators of CP110. If these areas can be addressed, I believe this study would provide an important addition to our understanding of ciliogenesis and linear ubiquitin modifications and be appropriate for publication in JCB.

Major issues:
1. LUBAC and PRPF8 are both shown to promote CP110 removal, but the functional relationship between them is not fully clear. How do the authors envision that PRPF8 'takes away' ubiquitinated CP110 from the mother centriole, and how does this model fit the observation that PRPF8 returns to the mother centriole at later stages of ciliogenesis while CP110 does not? It is also hard to reconcile the highly synchronous removal of CP110 and PRPF8 in the first 12h of serum starvation with the fact that PRPF8 subsequently returns at 24h while CP110 does not. One way to clarify the functional relationship between LUBAC and PRPF8 would be to examine PRPF8 localization following RNAi of LUBAC or removal of the LUBAC-interacting domains from PRPF8.

2. While it is clear that CP110 removal and ciliogenesis are defective when LUBAC or PRPF8 function is compromised, it would be helpful to provide additional evidence that these defects are due to direction regulation of CP110 by LUBAC/PRPF8 (versus LUBAC/PRPF8 acting at a separate/additional step in ciliogenesis that is in turn required for CP110 removal). Have the authors tested whether CP110 RNAi rescues the ciliogenesis defects seen in LUBAC/PRPF8 RNAi cells (as recently reported for loss of Cep78/EDD/VPRBP-mediated ubiquitination of CP110 - https://www.biorxiv.org/content/10.1101/2020.10.05.325936v2.full)? Similarly, the finding that several ciliogenesis/centriolar markers are unaffected in LUBAC RNAi cells provides support for the authors' model, but it would be helpful to examine additional markers such as MYO5A, EHD1, or IFT-B that act at an early stage in ciliogenesis.

3. Did the authors conduct rescue experiments for the zebrafish analyses of body axis curvature or KV cilia number? Such data would strengthen the in vivo analysis of LUBAC function and be valuable given that mouse or human LUBAC mutants do not have known ciliary defects.

Minor issues:
1. Some aspects of the introduction warrant minor modification. First, CP110 removal is presented as the first critical triggering event in ciliogenesis, but some studies (e.g. PMIDs 25686250, 30683896) suggest that other steps in ciliogenesis occur prior to or independent of CP110 removal. Second, while some background on CP110 removal is included, it would be helpful to cite publications linking Neurl4 and Cep78/EDD/DYRK2/VprBP to CP110 removal (PMIDs 22441691, 28242748 and preprint https://www.biorxiv.org/content/10.1101/2020.10.05.325936v2.full).

2. It would be helpful in the Discussion to comment on the mouse and human loss-of-function phenotypes reported for LUBAC and their relatedness to canonical ciliary defects. Similarly, it may help to further contextualize this study in relation to other reports on CP110/Cep97 ubiquitination, particularly since UBR5/EDD was recovered as a linear-ubiquitin-binding protein and is part of the Cep78/EDD/VPRBP pathway that mediates CP110 ubiquitination.

3. An anti-linear-Ub antibody is used throughout but no reference is cited and no validation of its selectivity for linear ubiquitin binding is included.

4. Did the authors perform rescue experiments for the PRPF8 RNAi studies?

5. Regarding Fig 3E, in immunoprecipitates of CP110, can high-molecular-weight forms of the protein be detected by anti-CP110 western blot? Can the authors comment on roughly what

fraction of total CP110 appears to be modified by linear Ub?

6. Regarding Fig 6A-D, the assessment of PRPF8 localization relative to other markers would benefit from higher-resolution imaging methods (eg SIM); alternatively, the statements regarding the precise localization of PRPF8 relative to other distal centriole markers should be tempered.

Reviewer #3 (Comments to the Authors (Required)):

In the manuscript entitled "LUBAC promotes ciliogenesis by regulating CP110 removal from the mother centriole." by Xiao-Lin Shen et al., authors have shown a new role of the ubiquitin E3 ligase complex LUBAC in the regulation of ciliognesis. They observed that knockdown of the LUBAC components in human and mouse cells leads to defects in ciligogenesis. In zebrafish, knockdown of HOIP (with a catalytic core of linear ubiquitination) caused defective left-right asymmetry. Mechanistically, they showed that LUBAC ubiquitinates CP110, a centrosome complex component, in cells. They also showed that PRPF8 as a linear ubiquitin chain receptor, which led to a working model that linearly ubiquitinated CP110 recruits PRPF8, which is important to remove the complex during ciliogenesis of which detailed mechanisms remain unclear.
The manuscript is well written, and the message is clear. However, there are some major issues, which need to be addressed before publication.

Major issues
1. For the catalytic inactive mutant of HOIP, the authors used a C299S/C702S/C871S/C874S mutant. These mutations lead to structural destruction of RING2 and it is not suitable to determine if the catalytic activity of HOIP is involved. It is necessary to use the HOIP C885S mutant instead (Fig 3C).
2. In the 1st page of the results section, line 4, they describe that LUBAC was localized to the centrosomes in interphase cells, but the data are not shown, nor citation is indicated. Please show these data since they are important for the study.
3. In Fig S6A, it is concluded that PRPF8 and CEP164 are colocalized, however, the colors are very similar in the figure panels, and it is not clearly visible. Please show them in different colors.
4. In Fig 5B, to conclude that CP110 recruits PRPF8 in a linear Ub-dependent manner, it is critical to compare LUBAC wt vs LUBAC-with HOIP C885S mutant. Current data sets provide only indirect evidence.
5. In Fig 7. The DeltaLUB mutant include deletions of two relatively large regions. By considering the functions of the domain indicated as "3" in ciliogenesis, it might be difficult to conclude that PRPF8-linera Ub binding regulates the shown process. To conclude this, it is necessary to use point mutants which specifically abolish linear Ub binding, which might be beyond the scope of this study. Thus, at least the authors should discuss alternative outcomes derived from these deletions.

Point-to-point responses to the reviewers' concerns

Reviewer #1

In this report Shen et al, provide extensive evidence supporting a role for the linear ubiquitin chain assembly complex (LUBAC) in the removal of the CP110-CEP97 from the mother centriole during ciliogenesis. They further propose that the splicing factor PRPF8 acts as a receptor for linear ubiquitinated CP110 which promote its removal thereby driving ciliogenesis.

This very interesting report, however, is not yet ready for the readership of the Journal of Cell Biology. Several important pieces of data are lacking.

1) The authors stated that the linear ubiquitin chain assembly complex (LUBAC) was localized to the centrosomes in interphase cells. No immunofluorescence data or a reference was provided to support this statement. When in interphase is LUBAC localized to the centrosome?

Response: We thank the reviewer for raising this important issue. Our research group has been working on exploring the function of the linear ubiquitin chain assembly complex (LUBAC) for many years (Wu et al., 2019). We previously transfected HeLa cells with the core catalytic subunit HOIP of LUBAC and found that the ectopically expressed HOIP was localized to the centrosomes in interphase cells (see revised Fig. S1 A). We further confirmed this observation in interphase RPE-1 cells (including G1, S/G2 phase) (see revised Fig. S1 B). Additionally, we also tried most of commercial antibodies and antibodies made by our lab to detect the localization of endogenous LUBAC components. Unfortunately, none of the antibodies against endogenous LUBAC components can be used for immunofluorescence.

2) The authors propose that LUBAC and CP110 form a robust complex, but this was shown by overexpression in 293 cells. Please provide evidence of the endogenous complex in RPE1 cells.

Response: We appreciate the reviewer for pointing out this issue. As suggested, we performed an endogenous immunoprecipitation experiment with anti-CP110 polyclonal antibody in RPE-1 cells. Our data showed that endogenous CP110 robustly interacts with the core catalytic subunit HOIP of LUBAC in RPE-1 cells (see revised Fig. 3 B), indicating that CP110 was presented in the same complex with LUBAC components.

3) LUBAC ubiquitylation of CP110 would be more convincing if the authors did this assay in vitro using purified components.

Response: We appreciate the reviewer's constructive suggestions and performed additional experiments accordingly. To detect the linear ubiquitination of CP110 *in vitro*, we expressed Flag-CP110 in HEK293T cells. Then the cell lysate was immunoprecipitated by anti-Flag M2 Affinity Gel, and the immunoprecipitant was eluted with 3×Flag peptide. Next, we performed the ubiquitination assay by incubating the eluted Flag-CP110 with E1, E2, and His-HOIP-RBR-LDD (Fu et al., 2021; Smit et al., 2012). Subsequently, the reaction mixtures were immunoprecipitated with anti-Flag M2 Affinity Gel, and the immunoprecipitates were detected by immunoblotting with the anti-linear ub antibodies. We found that Flag-CP110 was linearly ubiquitinated by His-HOIP-RBR-LDD (see revised Fig. 3 F), which indicated that CP110 is a substrate of LUBAC.

Reviewer #2

In this manuscript, Shen et al. examine the regulation of CP110, a protein that caps the mother centriole and acts as a key negative regulator of cilium assembly. Shen et al. report that the LUBAC ubiquitin ligase acts with PRPF8 to promote ciliogenesis via ubiquitination of CP110 and removal of CP110 from the mother centriole. Despite some issues and caveats noted below, overall this study provides convincing evidence that LUBAC assembles linear ubiquitin chains on CP110, that ubiquitinated CP110 is recognized by PRPF8, and that LUBAC and PRPF8 are necessary for CP110 removal and cilium assembly. The characterization and application of the CP110 allele that does not bind LUBAC or get removed from the mother centriole is particularly valuable. While prior studies have also examined CP110 regulation and identified CP110-modifying ubiquitin ligases (such as Neurl4 and EDD1-DDB1-VPRBP), the molecular pathways responsible for CP110 removal remain incompletely characterized, and the cellular functions of linear ubiquitin chains are also not fully understood.

The manuscript would be improved through further examination of the functional relationship between LUBAC and PRPF8, through evaluation of whether CP110 RNAi bypasses the roles of LUBAC/PRPF8 in ciliogenesis, and through better integration of the present findings with prior reports on regulators of CP110. If these areas can be addressed, I believe this study would provide an important addition to our understanding of ciliogenesis and linear ubiquitin modifications and be appropriate for publication in JCB.

Major issues:

1. LUBAC and PRPF8 are both shown to promote CP110 removal, but the functional relationship between them is not fully clear. How do the authors envision that PRPF8 'takes away' ubiquitinated CP110 from the mother centriole, and how does this model fit the observation that PRPF8 returns to the mother centriole at later stages of ciliogenesis while CP110 does not? It is also hard to reconcile the highly synchronous removal of CP110 and PRPF8 in the first 12h of serum starvation with the fact that PRPF8 subsequently returns at 24h while CP110 does not. One way to clarify the functional relationship between LUBAC and PRPF8 would be to examine PRPF8 localization following RNAi of LUBAC or removal of the LUBAC-interacting domains from PRPF8.

Response: We sincerely thank the reviewer for his/her interest in our study and for raising these important issues. We took them seriously and performed the experiment following the reviewer's suggestion. We examined PRPF8 localization following RNAi of LUBAC and found that knockdown of LUBAC did not affect the localization of PRPF8 during ciliogenesis (see revised Fig. 6, G and H), indicating the removal of PRPF8 is independent of LUBAC. Meanwhile, our original data showed that, unlike wild-type PRPF8, PRPF8 mutant failed to bind to the linear ubiquitin chains, could not rescue ciliogenesis and CP110 removal in PRPF8-depleted cells (see revised Fig. 7 D-F), suggesting that PRPF8 regulates CP110 removal and ciliogenesis depending on its binding to the linear ubiquitin chains. Therefore, based on these new and our original data, we proposed our model as following: **(1)** LUBAC catalyzes the linear ubiquitination of CP110, which is required for CP110 removal from the mother centriole in ciliogenesis (see revised Fig. 3 and 4). **(2)** PRPF8 acts as the receptor for the linear ubiquitin chains to regulate CP110 removal in ciliogenesis (see revised Fig. 5-7). (3) The removal of PRPF8 is independent of LUBAC (see

revised Fig. 6, G and H). Thus, we proposed that PRPF8 'takes away' the linearly ubiquitinated CP110 catalyzed by LUBAC from the mother centriole and subsequently promotes ciliogenesis.

Additionally, our data also showed that at later stages of ciliogenesis, PRPF8 returns to the mother centriole while CP110 does not (see revised Fig. S7, B and C). Since several research groups showed that CP110 is degraded upon it removed from the mother centriole in RPE-1 cells (Cao et al., 2012; Huang et al., 2018), which could be the reason why CP110 can't return to the mother centriole with PRPF8 at the late stage of ciliogenesis.

2. While it is clear that CP110 removal and ciliogenesis are defective when LUBAC or PRPF8 function is compromised, it would be helpful to provide additional evidence that these defects are due to direction regulation of CP110 by LUBAC/PRPF8 (versus LUBAC/PRPF8 acting at a separate/additional step in ciliogenesis that is in turn required for CP110 removal). Have the authors tested whether CP110 RNAi rescues the ciliogenesis defects seen in LUBAC/PRPF8 RNAi cells (as recently reported for loss of Cep78/EDD/VPRBP-mediated ubiquitination of CP110 - https://www.biorxiv.org/content/10.1101/2020.10.05.325936v2.full)? Similarly, the finding that several ciliogenesis/centriolar markers are unaffected in LUBAC RNAi cells provides support for the authors' model, but it would be helpful to examine additional markers such as MYO5A, EHD1, or IFT-B that act at an early stage in ciliogenesis.

Response: We appreciate the reviewer for raising these important issues and giving us valuable suggestions. As suggested by the reviewer, we depleted CP110 in LUBAC- or PRPF8-knockdown cells and detected ciliogenesis with the ciliary marker (Ac-tubulin). The result showed that knockdown of CP110 significantly rescued the defect of ciliogenesis caused by LUBAC or PRPF8 depletion, indicating that both LUBAC and PRPF8 promote ciliogenesis by regulating CP110 removal from the mother centriole (see revised Fig. 2, G and H; and Fig. 5, F and G).

In addition, we examined MYO5A and IFT20, which are required for ciliary vesicle (CV) formation in the initial stage of ciliogenesis. We found that MYO5A and IFT20 were unaffected in LUBAC-depleted cells (see revised Fig. 2 D; and Fig. S3 D), suggesting that LUBAC is not necessary for CV formation. An additional marker suggested by the reviewer is EHD1. We tried to find the proper antibody to test the localization of EHD1. Unfortunately, the anti-EHD1 antibody (NBP1-95580, NOVUS) used in the previous study is discontinued (Wu et al., 2018), and another anti-EHD1 polyclonal antibody (24657-1-AP, proteintech) didn't work for the immunofluorescence detection of EHD1.

3. Did the authors conduct rescue experiments for the zebrafish analyses of body axis curvature or KV cilia number? Such data would strengthen the in vivo analysis of LUBAC function and be valuable given that mouse or human LUBAC mutants do not have known ciliary defects.

Response: Thanks for raising this important issue. Following the reviewer's suggestion, we conducted the rescue experiment of body axis curvature by co-injecting the translation blocking morpholino (aMO) and the aMO-resistant form of zebrafish *rnf31* mRNA (*rnf31*-re-zmRNA) into zebrafish. As expected, the body axis curvature was significantly rescued by *rnf31*-re-zmRNA compared with aMO-knockdown morphants (see revised Fig. S2 D).

Minor issues:

1. Some aspects of the introduction warrant minor modification. First, CP110 removal is presented as the first critical triggering event in ciliogenesis, but some studies (e.g. PMIDs 25686250, 30683896) suggest that other steps in ciliogenesis occur prior to or independent of CP110 removal. Second, while some background on CP110 removal is included, it would be helpful to cite publications linking Neurl4 and Cep78/EDD/DYRK2/VprBP to CP110 removal (PMIDs 22441691, 28242748 and preprint https://www.biorxiv.org/content/10.1101/2020.10.05.325936v2.full).

Response: Thank the reviewer for pointing out these issues and giving us a chance to correct our negligence. We completely accepted reviewer's comment and rephrased our statement '**it is generally accepted that loss of CP110 on the mother centriole is a crucial event at the onset of ciliogenesis**' in our revised manuscript. Additionally, we learned a lot and cited these elegant papers (Goncalves et al., 2021; Hossain et al., 2017; Li et al., 2012) in our revised manuscript (*Lines 71 -75*).

2. It would be helpful in the Discussion to comment on the mouse and human loss-of-function phenotypes reported for LUBAC and their relatedness to canonical ciliary defects. Similarly, it may help to further contextualize this study in relation to other reports on CP110/Cep97 ubiquitination, particularly since UBR5/EDD was recovered as a linear-ubiquitin-binding protein and is part of the Cep78/EDD/VPRBP pathway that mediates CP110 ubiquitination.

Response: We sincerely thank the reviewer for his/her interest in our study and giving us a valuable suggestion. As suggested by the reviewer, we added the comment on human or mouse LUBAC loss-of-function phenotypes associated with canonical ciliary defects in the discussion section of our revised manuscript (*Lines 377-382*).

Meanwhile, the reviewer raised a very interesting and constructive point that our study may be related to other reports on CP110 ubiquitination. The E3 ligase UBR5/EDD was recently reported to mediate CP110 ubiquitination degradation in ciliogenesis (Goncalves et al., 2021; Hossain et al., 2017). In our original Fig. S5 A, we identified that UBR5/EDD might be a linear ubiquitin chains binding candidate protein by MS. Based on previous reports and our data, UBR5/EDD is possibly recruited by linear ubiquitin chains of CP110 to facilitate the degradation of CP110, after the removal of the linearly ubiquitinated CP110 by PRPF8 from the mother centriole. We have discussed this important issue in our revised manuscript (*Lines 366-372*). It is also interesting to investigate this issue in the future.

3. An anti-linear-Ub antibody is used throughout but no reference is cited and no validation of its selectivity for linear ubiquitin binding is included.

Response: We apologized for our negligence about the reference citations of the anti-linear-Ub antibody. The anti-linear-Ub antibody used in our study is kindly gifted from Dr. Vishva M. Dixit, and its specificity for linear ubiquitin binding was validated by many assays (Matsumoto et al., 2012). This anti-linear ubiquitin antibody was used in many other studies after it was developed (Keusekotten et al., 2013; Rivkin et al., 2013; Rodgers et al., 2014). Our research group also tested its specificity for linear ubiquitin in a previous work (Wu et al., 2019). We have cited these references in our revised manuscript.

4. Did the authors perform rescue experiments for the PRPF8 RNAi studies?

Response: Many thanks for raising this important issue and apologized for our undetailed descriptions in our original manuscript. In our original Fig. 7 D and E, we perform rescue experiments in PRPF8-depleted cells with wild-type PRPF8 or ΔLUB mutant. The results showed that, unlike wild-type PRPF8, ΔLUB mutant failed to promote CP110 removal from the mother centrioles after serum starvation (see revised Fig. 7, D and E). Importantly, compared to wild-type PRPF8, ΔLUB mutant could not rescue the defects of ciliogenesis caused by depletion of PRPF8 (see revised Fig. 7, D and F).

5. Regarding Fig 3E, in immunoprecipitates of CP110, can high-molecular-weight forms of the protein be detected by anti-CP110 western blot? Can the authors comment on roughly what fraction of total CP110 appears to be modified by linear Ub?

Response: Thanks for raising these important issues. We took it seriously and carefully checked the original western blot film stained by anti-CP110 antibody in our original Fig. 3 E and observed the signal of high-molecular-weight forms. However, compared to control, depletion of HOIP did not reduce the signal of high-molecular-weight forms (Fig. 1 for reviewers). We speculated that these high-molecular-weight forms may contain other linkage types of polyubiquitin chains of CP110 or non-specific signals. Therefore, due to the valence of anti-CP110 antibody and other technical limitations, it is hard to detect and calculate the percentages of linearly ubiquitinated CP110.

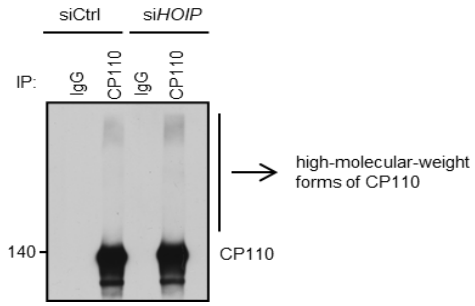

Fig. 1 The original western blot film stained by anti-CP110 antibody in our original Fig. 3 E.

6. Regarding Fig 6A-D, the assessment of PRPF8 localization relative to other markers would benefit from higher-resolution imaging methods (eg SIM); alternatively, the statements regarding the precise localization of PRPF8 relative to other distal centriole markers should be tempered.

Response: Thanks for the reviewer's constructive suggestions. Due to the implementation of measures to limit spread of COVID-19, it is difficult to find the higher-resolution microscopy to take SIM images. Alternatively, following the reviewer's suggestion, we have rephrased the description of the precise localization of PRPF8 in our revised manuscript (*Lines 293-300*).

Reviewer #3

In the manuscript entitled "LUBAC promotes ciliogenesis by regulating CP110 removal from the mother centriole." by Xiao-Lin Shen et al., authors have shown a new role of the ubiquitin E3 ligase complex LUBAC in the regulation of ciliognesis. They observed that knockdown of the LUBAC components in human and mouse cells leads to defects in ciligogenesis. In zebrafish, knockdown of HOIP (with a catalytic core of linear ubiquitination) caused defective left-right asymmetry. Mechanistically, they showed that LUBAC ubiquitinates CP110, a centrosome complex component, in cells. They also showed that PRPF8 as a linear ubiquitin chain receptor, which led to a working model that linearly ubiquitinated CP110 recruits PRPF8, which is important to remove the complex during ciliogenesis of which detailed mechanisms remain unclear.

The manuscript is well written, and the message is clear. However, there are some major issues, which need to be addressed before publication.

Major issues

1. For the catalytic inactive mutant of HOIP, the authors used a C299S/C702S/C871S/C874S mutant. These mutations lead to structural destruction of RING2 and it is not suitable to determine if the catalytic activity of HOIP is involved. It is necessary to use the HOIP C885S mutant instead (Fig 3C).

Response: We sincerely thank the reviewer for raising this important issue. Following the reviewer's suggestion, we constructed the HOIP C885S mutant (Lafont et al., 2018; Smit et al., 2012; Stieglitz et al., 2012) and further tested its catalytic activity on CP110 by performing the ubiquitination assay. Our result showed that, compared to wild-type HOIP, the HOIP C885S mutant could not linearly ubiquitinate CP110 (see revised Fig. 3 D), indicating that CP110 is a catalytic substrate of LUBAC.

2. In the 1st page of the results section, line 4, they describe that LUBAC was localized to the centrosomes in interphase cells, but the data are not shown, nor citation is indicated. Please show these data since they are important for the study.

Response: We thank the reviewer for raising this important issue. Our research group has been working on exploring the function of the linear ubiquitin chain assembly complex (LUBAC) (Wu et al., 2019). We previously transfected HeLa cells with the core catalytic subunit HOIP of LUBAC and found that the ectopically expressed HOIP was localized to the centrosomes in interphase cells (see revised Fig. S1 A). We further confirmed this observation in interphase RPE-1 cells (including G1, S/G2 phase) (see revised Fig. S1 B). Additionally, we also tried most of commercial antibodies and antibodies made by our lab to detect the localization of endogenous LUBAC components. Unfortunately, none of the antibodies against endogenous LUBAC components can be used for immunofluorescence.

3. In Fig S6A, it is concluded that PRPF8 and CEP164 are colocalized, however, the colors are very similar in the figure panels, and it is not clearly visible. Please show them in different colors.

Response: Many thanks to the reviewer for his/her keen observation and suggestion. We have modified the colors in the graph (see revised Fig. S7 A).

4. In Fig 5B, to conclude that CP110 recruits PRPF8 in a linear Ub-dependent manner, it is critical to compare LUBAC wt vs LUBAC-with HOIP C885S mutant. Current data sets provide only indirect evidence.

Response: We are grateful for the reviewer's suggestion and performed the experiment accordingly. Our results showed that the linear-ubiquitinated CP110 by LUBAC, but not non-linear-ubiquitinated CP110 (co-expressed with HOIP C885S mutant), could efficiently bind to endogenous PRPF8 (see revised Fig. 5 B). These data suggested that CP110 interacts with PRPF8 in a linear Ub-dependent manner.

5. In Fig 7. The DeltaLUB mutant include deletions of two relatively large regions. By considering the functions of the domain indicated as "3" in ciliogenesis, it might be difficult to conclude that PRPF8-linera Ub binding regulates the shown process. To conclude this, it is necessary to use point mutants which specifically abolish linear Ub binding, which might be beyond the scope of this study. Thus, at least the authors should discuss alternative outcomes derived from these deletions.

Response: We appreciate the reviewer for pointing out this important issue and giving us valuable suggestions. We have tried to find the minimal linear Ub binding regions of PRPF8. Since PRPF8 doesn't contain the UBAN domain (the conserved linear ubiquitin chain-specific binding domain), we thus mapped the interaction regions of PRPF8 with GST-Ub4. The results showed that both residues 1301-1669 and residues 2234-2335 of PRPF8 could bind to the linear ubiquitin chains (see revised Fig. 7 B). Furthermore, both deletions of residues 1301-1669 and 2234-2335 (ΔLUB) of PRPF8 compromised its ability to bind to GST-Ub4, while alone deletion of residues 1301-1669 (Δ1301-1669) or residues 2234-2335 (Δ2234-2335) could not abolish the ability of PRPF8 binding to GST-Ub4 (Fig 2 for reviewers), indicating that these two regions are simultaneously required for PRPF8 binding to linear ubiquitin chains. Therefore, it is difficult to find out point mutants that specifically abolish linear Ub binding of PRPF8. Alternatively, we discussed probable outcomes derived from these deletions in our revised manuscript (*Lines 392-401*).

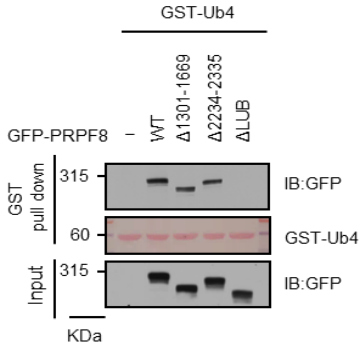

Fig. 2 GFP-vector (–), GFP-PRPF8 WT, Δ1301-1669 mutant, Δ2234-2335 mutant, or ΔLUB mutant was transfected into HEK293T cells. The cell lysates were pulled down by GST-Ub4, and then GFP proteins were detected by anti-GFP antibody. GST-Ub4 was stained by Ponceau S.

September 23, 2021

Re: JCB manuscript #202105092R

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 "LUBAC promotes ciliogenesis by regulating CP110 removal from the mother centriole." The manuscript has been seen by the original reviewers whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

You will see that Reviewer #2 asks to confirm with a cilia-specific marker that the cilia observed in CP110 RNAi experiments are not elongated centrioles. There are also a few minor comments that can be easily addressed by text revisions.

Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor we are open to one additional short round of revision. Please note that we expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining reviewer comments.

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

Sincerely,

Maxence Nachury, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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

In this report Shen et al, provide extensive evidence supporting a role for the linear ubiquitin chain assembly complex (LUBAC) in the removal of the CP110-CEP97 from the mother centriole during ciliogenesis. They further propose that the splicing factor PRPF8 acts as a receptor for linear ubiquitinated CP110 which promote its removal thereby driving ciliogenesis.

This very interesting report, however, is suitable for the readership of the Journal of Cell Biology with one major revision.

The authors must revise the following statement (see below) in their manuscript so that it reflects exactly what is shown in Figure S1, A and B.

"We recently found that the linear ubiquitin chain assembly complex (LUBAC) was localized to the centrosomes in interphase cells (Fig. S1, A and B). "

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

In this revised manuscript, Shen et al. have addressed many of the reviewer concerns and significantly improved their manuscript. I am glad to see the efforts they went to, and I now feel the manuscript is nearly ready for publication in JCB and would be of high interest to JCB's readership. There are however some minor issues noted below that I think should be attended to in the final manuscript.

1. I am glad to see the new data in response to major point 1 raised previously (Fig. 6G-H). Specifically, the authors now show that PRFP8 is recruited to the basal body in serum-fed cells in a LUBAC-independent manner. These data also show that PRPF8 is removed in response to serum starvation even upon RNAi of LUBAC (a condition that blocks CP110 ubiquitination and removal). Thus, both the recruitment and removal of PRPF8 at the mother centriole appear to occur independently of LUBAC-mediated CP110 ubiquitination. This finding in turn raises the question of how these PRPF8 recruitment/removal events are triggered and whether they have functional significance independent of the proposed role of PRPF8 as a receptor for ubiquitinated CP110. It is likewise unclear why PRPF8 is initially removed from the basal body upon serum starvation only to return at later stages of ciliogenesis. While definitive answers to these questions are likely beyond the scope of the present paper, I believe it would nonetheless be helpful to further discuss or clarify these aspects with respect to the overall model that is proposed.

2. The newly added data in Figs. 2G-H and 5F-G that show CP110 RNAi rescues ciliogenesis in cells depleted of LUBAC or PRPF8 is a valuable addition and supports the central model of the manuscript. I am reluctant to ask for further experimental work, but did the answers assess ciliation under these conditions using a cilia-specific marker such as Arl13b? This point is relevant because CP110 depletion has previously been shown to cause a centriole elongation phenotype; these elongated centrioles are marked by acetylated tubulin (the marker that appears to have been used for these figures) and can be easily misidentified as cilia (see PMIDs 19481458, 19481460, 21620453). Assessing the CP110 RNAi rescue phenotype with a ciliary membrane marker would confirm that these are bona fide cilia.

3. The revision added on lines 377-379 is helpful but the phrasing/grammar is a bit unclear.

Reviewer #3 (Comments to the Authors (Required)):

The authors have fully addressed my comments. I recommend accepting their manuscript for publication.

Point-to-point responses to the reviewers' concerns

Reviewer #1

In this report Shen et al, provide extensive evidence supporting a role for the linear ubiquitin chain assembly complex (LUBAC) in the removal of the CP110-CEP97 from the mother centriole during ciliogenesis. They further propose that the splicing factor PRPF8 acts as a receptor for linear ubiquitinated CP110 which promote its removal thereby driving ciliogenesis.

This very interesting report, however, is suitable for the readership of the Journal of Cell Biology with one major revision.

The authors must revise the following statement (see below) in their manuscript so that it reflects exactly what is shown in Figure S1, A and B.

"We recently found that the linear ubiquitin chain assembly complex (LUBAC) was localized to the centrosomes in interphase cells (Fig. S1, A and B). "

Response： We are grateful to the reviewer for the positive assessment and apologized for our imprecise descriptions of Fig. S1, A and B in our manuscript. Following the reviewer's suggestion, we have revised the statement to "We transfected Hela cells with the core catalytic subunit HOIP of the linear ubiquitin chain assembly complex (LUBAC) and found that HOIP was localized to the centrosomes in interphase cells (Fig. S1 A). We further confirmed this observation in human retinal pigment epithelial (RPE-1) cells (Fig. S1 B)" in our revised manuscript (Lines 115-119).

Reviewer #2

In this revised manuscript, Shen et al. have addressed many of the reviewer concerns and significantly improved their manuscript. I am glad to see the efforts they went to, and I now feel the manuscript is nearly ready for publication in JCB and would be of high interest to JCB's readership. There are however some minor issues noted below that I think should be attended to in the final manuscript.

1. I am glad to see the new data in response to major point 1 raised previously (Fig. 6G-H). Specifically, the authors now show that PRFP8 is recruited to the basal body in serum-fed cells in a LUBAC-independent manner. These data also show that PRPF8 is removed in response to serum starvation even upon RNAi of LUBAC (a condition that blocks CP110 ubiquitination and removal). Thus, both the recruitment and removal of PRPF8 at the mother centriole appear to occur independently of LUBAC-mediated CP110 ubiquitination. This finding in turn raises the question of how these PRPF8 recruitment/removal events are triggered and whether they have functional significance independent of the proposed role of PRPF8 as a receptor for ubiquitinated CP110. It is likewise unclear why PRPF8 is initially removed from the

basal body upon serum starvation only to return at later stages of ciliogenesis. While definitive answers to these questions are likely beyond the scope of the present paper, I believe it would nonetheless be helpful to further discuss or clarify these aspects with respect to the overall model that is proposed.

Response: Many thanks for the reviewer's encouraging comments on our manuscript. We agree with the reviewer on these points about PRPF8. In our study, we uncovered an essential role of PRPF8 in CP110 removal during ciliogenesis. Our data demonstrated that, at the initial stage of ciliogenesis, PRPF8 serves as the receptor for linear ubiquitin chains of CP110 and disappears from the mother centriole to promote CP110 removal. While our futher results showed that the recruitment and removal of PRPF8 at the mother centriole are not regulated by LUBAC, suggesting that additional mechanisms regulate the recruitment and removal of PRPF8. In addition, why PRPF8 returns to the ciliary base at the later stage of ciliogenesis, this is an interesting phenomenon. These issues are very valuable and will be further investigated in our future studies. Following the reviewer's suggestion, we have discussed these remained issues in our revised manuscript (Lines 390-401).

2. The newly added data in Figs. 2G-H and 5F-G that show CP110 RNAi rescues ciliogenesis in cells depleted of LUBAC or PRPF8 is a valuable addition and supports the central model of the manuscript. I am reluctant to ask for further experimental work, but did the answers assess ciliation under these conditions using a cilia-specific marker such as Arl13b? This point is relevant because CP110 depletion has previously been shown to cause a centriole elongation phenotype; these elongated centrioles are marked by acetylated tubulin (the marker that appears to have been used for these figures) and can be easily misidentified as cilia (see PMIDs 19481458, 19481460, 21620453). Assessing the CP110 RNAi rescue phenotype with a ciliary membrane marker would confirm that these are bona fide cilia.

Response: We appreciate the reviewer for raising this important issue and providing the valuable suggestion. In our original Fig 2G and Fig 5F, we indeed stained primary cilia with acetylated tubulin. Following the reviewer's suggestion, we further assessed the CP110 RNAi rescue phenotype by staining with a ciliary membrane marker ARL13B. Similar to our original results, the knockdown of CP110 significantly rescued the defect of ciliogenesis in LUBAC or PRPF8-depleted cells (see revised Fig. 2, G and H; and Fig. 5, F and G). These data confirmed that these cilia in CP110 RNAi experiments were bona fide cilia.

3. The revision added on lines 377-379 is helpful but the phrasing/grammar is a bit unclear.

Response: Many thanks to the reviewer for his/her keen observation and suggestion. We have rephrased the statement to "Although there is no evidence that human beings with LUBAC deficiency suffer from ciliopathies, our study might bring a cue for this issue, which should be concerned in future research." in our revised manuscript (Lines 378-380).

Reviewer #3

The authors have fully addressed my comments. I recommend accepting their manuscript for publication.

October 5, 2021

RE: JCB Manuscript #202105092RR

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 "LUBAC promotes ciliogenesis by regulating CP110 removal from the mother centriole". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). We also ask that you please add representative micrographs of the ARL13B stainings used for the quantifications shown in Figures 2G and 5F.

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 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.

2) Figures limits: Articles may have up to 10 main text figures.

3) 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.

4) 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, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Title: We suggest changing the beginning of the title to "LUBAC regulates ciliogenesis by promoting..." to more accurately reflect the findings of the study.

6) 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."

7) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or gifted to you by other investigators (and provide references where appropriate). Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary. If antibodies are not commercial please add a reference citation if possible.

8) 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 reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures and 10 videos. You currently exceed this limit but, in this case, we will be able to give you the extra space but please 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. Please include one brief sentence per item.

11) 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.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests

are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

14) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

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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,

Maxence Nachury, PhD

Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology