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Cep57 and Cep57L1 maintain centriole engagement in interphase to ensure centriole duplication cycle

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July 22, 2020

Re: JCB manuscript #202005153

Prof. Daiju Kitagawa
University of Tokyo
Hongo 7-3-1
Bunkyo-ku, Tokyo 113-0033
Japan

Dear Prof. Kitagawa,

Thank you for submitting your manuscript entitled "Cep57 and Cep57L1 maintain centriole engagement in interphase to ensure centriole duplication cycle." 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.

Overall, the reviewers were enthusiastic about the study but raised several concerns which will need to be addressed before this paper would be ready for publication in JCB. The main concern shared by reviewers is regarding the efficiency of siRNA knockdowns. Reviewer 1 requests data validating the extent of the knockdowns and asks whether depletion efficiency correlates with phenotype. Reviewer 2 raises more significant concerns regarding potentially incomplete knockdown and requests analysis of CRISPR knockouts to rule out possible partial recruitment of Cep152 which may lead to premature centriole disengagement. While we agree that validation with CRISPR knockouts would help support the conclusions we also understand that generating these lines may not be feasible in a short time frame. Thus, we will not require these for publication in JCB. However, in the absence of CRISPR knockouts we ask that you show data for all the siRNA knockouts by Western and IF demonstrating over 95% knockdown efficiency as well as tone down the conclusions to consider the effects of incomplete knockdown. Please be sure to also include a point-by-point rebuttal for all the items raised by the reviewers.

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.

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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.***

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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,

Yixian Zheng, Ph.D.
Monitoring Editor
Journal of Cell Biology

Dan Simon, Ph.D.
Scientific Editor
Journal of Cell Biology

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

Cep57 and Cep57L1 maintain centriole engagement in interphase to ensure centriole duplication cycle

Ito et al. report in this manuscript that CEP57 together with its paralogue CEP57L is involved in connecting of the daughter centriole to the mother from S phase during G2. This is very interesting since proteins with such a function have not been identified. However, the manuscript does not characterize how CEP57 and CEP57L1 are achieving this linkage. This is in my opinion a slight disadvantage of the paper. Instead, the authors focus on the consequences of premature centriole

disengagement in interphase. They convincingly show that centriole disengagement in G2 trigger PLK1-dependent maturation of the daughter and in addition, centriole duplication. As a consequence of this, cells enter mitosis with more than the usual (two) active centrosomes and form multi-polar spindles leading to chromosome misalignment.

Considering that this manuscript identifies the first molecules involved in centriole interphase centriole cohesion, I strongly believe that it is suitable for publication in J Cell Biol. I have listed a number of specific points that the authors should address.

Specific points
1. Fig. 1C-E: Double depletion of CEP57 and CEP57L1 causes centriole disengagement and triggers centriole amplification. Fig. 1C shows two centriole pairs: two are close together, two are well separated. I assume that the pairs represent mother and daughter. Why are the two pairs not kept together by the centrosome linker? Why is the separation of mother and daughter relative moderate (just above 0.75 µm - the centrioles in siControl in Fig. 1C have nearly the same distance. However, I assume that these are the two mother centrioles kept together by the linker). The phenotype is with 16% relatively moderate. Is this explained by the poor depletion efficiency of the two proteins? What is the depletion efficiency and does it correlate with the phenotype - please quantify (a single picture in Fig. 5C is not sufficient).
2. In Fig. 1C centriole pairs stay together upon co-depletion of CEP57 and CEP57L1. This picture is different to Fig. 3C (middle). The 4 centrioles are well separated. What is the difference?
3. It might be worth to study Sas-6 localisation of disengaged CEP57 and CEP57L1 depleted centrioles in S/G2 phase.
4. The authors should show the mitotic localisation of CEP57 and CEP57L1. The single telophase picture in Fig. 5A suggests that CEP57L1 is not or much less associated with mitotic centrosomes. This may explain why single depletion of CEP57 in mitosis is sufficient to trigger centriole disengagement (or the mechanism is via PCNT).
5. The authors should improve the discussion. The authors should discuss the molecular mechanism of centriole disengagement and the targets of PLK1. Why do cells have two proteins that function redundantly in this process? How is the daughter centriole formed in the absence of CEP152, a PLK4 adaptor. CEP57 may have several functions: In interphase together with CEP57L1 it ensures centriole engagement. In mitosis it interacts with PACT of PCNT and via PCNT keeps the two centrosomes together although with an increase in distance.
6. Fig. 9: The centriole disengagement picture shows a line connecting mother with daughter centrioles. I assume that this is PCNT. Readers may misunderstand it as the centrosome linker.

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

The manuscript by Ito et al reported the redundant function of cep57 and cep57L in centrosome biogenesis. The same pair of genes have been examined previously by two other groups, both of which showed that cep57/57L is required for canonical centriole duplication through recruiting an essential duplication factor cep152 to the mother centriole. Here, Ito et al uncovered a novel phenotype associated with loss of cep57/57L, which is in stark contrast to what was reported before. Ito et al did not see centriole duplication failure upon knockdown of cep57/57L; instead, they saw relatively normal initiation of centriole duplication. However, in the absence of cep57/57L, Ito et al further saw that the newborn daughter centriole that normally should form a tight association (or engagement) with the mother centriole can become prematurely disengaged before mitosis, a phenotype that would unlock the block for centriole reduplication, leading to centriole/centrosome amplification and mitotic errors. As the highlight of the story, the authors conclude that cep57/57L

function together to maintain centriole engagement, ensuring the block for centriole amplification. This function, however, is completely opposite to what has been reported.

The authors made little to no effort to resolve the discrepancy mentioned above, which will be a major concern for the scientific community if not addressed. With the current data, I am a bit skeptical about the new phenotype highlighted here (see the reasons a-e listed below), but there are ways to clear the doubt (see the essential experiments 1 below).

a. All 3 labs, including the one here, observed a similar requirement of cep57/57L for cep152 recruitment. As cep152 is essential for centriole duplication, centriole assembly should be affected by loss of cep57/57L, but that was not reported in this manuscript. I therefore worry that perhaps it is the incomplete phenotype, rather than null phenotypes, that was observed, which could potentially be a result from partial knockdown of cep57/57L using RNAi.

b. Consequently, it is possible that the premature centriole disengagement is also an intermediate phenotype resulted from partial knockdown of cep57/57L (see d and e for details).

c. Aziz et al (2018) showed that there is a difference in severity of phenotype for Cep57-/- mice as opposed to Cep57T/T mice which model a mutation found in human mosaic-variegated aneuploidy syndrome. Whereas the homozygous null mutation was embryonic lethal, mice with the disease mutation lived until after birth and showed supernumary centrosomes and premature disengagement. Given this evidence it is important to fully knockout Cep57 and cep57L to clarify if/what phenotype differences there may be between partial and complete protein loss.

d. One important notion about centriole assembly and centriole engagement is that they clearly share some common molecular components. For example, cep152, plk4, STIL and Sas6 are known to form the basic scaffold (as part of the cartwheel) upon which all vertebrate centrioles are built. Cep152 is also the main component of the PCM at the mother centriole where it recruits plk4/STIL/sas6 for centriole duplication. After duplication, the cep152 that is part of the PCM of the mother centriole would become embedded in the newborn centriole as part of the cartwheel and therefore, intuitively, can provide a direct physical connection between the newborn and mother centrioles. In this sense, centriole engagement and centriole duplication are functionally coupled, at least in part through cep152. It is thus entirely possible to create a situation where there is enough cep152 to drive initial centriole assembly but not enough to fully occupy the PCM for maintaining stable engagement, leading to premature detachment of newborn centrioles from mother centrioles.

e. Based on all these concerns mentioned above, the authors need to conduct essential experiments to confirm that the premature centriole disengagement seen in their assay is NOT due to partial reduction of cep152 or partial knockdown of cep57/57L. That is, I worry that when cep57/57L is fully depleted, a null phenotype where no cep152 is recruited and no canonical duplication can occur may become dominant. If so, the stated conclusion would be incorrect.

Essential experiments:
1. The authors must generate CRISPR knockout cells (cep57ko, cep57Lko, and cep57/57L double ko) either in clonal lines or mixed population and repeat the phenotype characterization in these KO cells. It is strongly suggested that all knockout cells should be made in p53-/- background to avoid stress response evoked by centrosome loss and the associated mitotic insults. If the double KO cells are not viable, one can still examined the phenotype before cells die using inducible CRISPR. Null or knockout analysis is now the gold standard for centrosome research. The key is to look for

null phenotypes by all means.

Other issues:
Figure 1:
Panel C:
• Cep 192 is not a good marker for the chosen experiment, as it is recruited to daughter centrioles specifically in G2. Staining for this marker clearly biases the count towards interphase cells in G2, unfairly excluding S phase cells. However, the quantification in Panels E and F do not indicate counting only cells in G2 phase, but rather all of interphase.
• Authors should use a different marker or indicate they are only counting G2 cells.

Figure 2:
Panel E:
• This figure requires a y-axis label and clarification on what is being quantified.

Figure 3:
Panel A and B:
• POC5 is recruited to newborn centrioles in G2, and is thus a marker for daughter centrioles only in G2. Disengagement, while premature, still takes time to occur, so cells with fully disengaged centrioles as judged by distance are likely at the later stages of the cell cycle, e.g. G2, which explains why POC5 is present at most of these centrioles. But S-phase cells with prematurely disengaged centrioles are mostly missed by this staining.
• Authors should quantify disengagement in S and G2 phase separately, as is done in Panel D

Figures 3 and 4:
Panel C and D (Figure 3) / Panel E-H (Figure 4):
• Pericentrin is a tricky marker, as even newborn centrioles that assemble de novo will have small but clear pericentrin foci at the start of growth before acquiring MTOC competence, and will recruit significantly more as part of the PCM only after centriole-to-centrosome conversion has occurred. That is, there should not be any PCNT-negative centrioles in cells. I don't know how the authors took the images. Maybe those PCNT-negative centrioles are not real centrioles, but centrin satellites that were misjudged as centrioles. Additional markers are needed to confirm the presence of real centrioles.
• To show black and white results for MTOC competence or centrosome maturation, authors should stain for a marker such as Cnap1/cep250, but not pericentrin

Figure 5:
Given the localization of Cep57 to the kinetochore as well as the centrosome it would be important for authors to examine if Cep57L1 is also present at the kinetochore.

Figure 6:
The diagram in Figure E does not depict the redundancy and effect of combined knockdown of both Cep57 and Cep57L1, particularly on cep152 recruitment. Perhaps authors can include an additional colored arrow to indicate effect of the combined knockdown.

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

In this MS, Ito and colleagues describe Cep57L1 as a component that together with Cep57 maintains centrioles engaged in interphase. In contrast to Cep57, Cep57L1 is not required for

centriole engagement in mitosis, implying an interphase specific function for Cep57L1. In interphase, Cep57 and Cep57L1 maintained engaged centrioles independently of Plk1. Prematurely disengaged centrioles upon Cep57/Cep57L1 co-depletion acquire pericentriolar material proteins (PCM) prior to mitosis and convert into functional centrosomes that nucleate microtubules. This leads to the formation of multi-polar spindles and chromosome mis-segregation, highlighting the importance for this regulation.

Overall, I found the study very interesting. The mechanisms that keep centrioles engaged in interphase are still poorly understood and this paper now shows that Cep57L1 and Cep57 cooperatively control this process. The experiments are of high quality and the authors provide a comprehensive analysis of Cep57/Cep57L1 depleted cells. However, I miss a clear model to explain why Cep57L1 is specifically involved in centriole engagement in interphase but not mitosis.

Specific points:
1. I found quite puzzling that Cep57 but not Cep57L1 is required for centriole engagement in mitosis. A simple explanation could be that Cep57L1 is degraded or not present at centrioles in mitosis, explaining the requirement for both proteins in interphase but only Cep57 in mitosis. This could be easily tested by analyzing Cep57L1 protein levels and centriole association at different stages of the cell cycle.

2. In figure 5B, the authors analyze Cep57L1 co-localization with PCNT and inferred that Cep57L1 forms a ring-like structure at the mother centriole that is similar to the ring formed by Cep57. However, this conclusion was based on previously published data for Cep57 (Watanabe et al. 2019). Ideally, the authors should co-stain Cep57 and Cep57L1 for better comparison. This should be feasible considering that rabbit and mouse antibodies are available for Cep57L1 and Cep57, respectively. This will also be important considering that Cep57L1 centriolar levels increase upon Cep57 depletion. Is the location of Cep57L1 rings also changed upon Cep57 depletion?

Minor points
1. Please check the y-axis of the graph shown in Figure 2E. Do the authors mean cells instead of percentage of cells?

2. The authors should cite the recent publication showing that Cep57 and Cep57L1 recruits Cep63-Cep152 complex for centriole biogenesis (Zhao et al., JCS 2020).

(point-by-point response to the comments from the reviewers)

We thank all the reviewers of our original manuscript for their critical reading and useful and constructive comments (typed in blue), which we fully addressed in the revised manuscript with new data (answers typed in black). Following their suggestions, we extensively performed the experiments and substantially altered the manuscript, as detailed below.

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

Cep57 and Cep57L1 maintain centriole engagement in interphase to ensure centriole duplication cycle

Ito et al. report in this manuscript that CEP57 together with its paralogue CEP57L is involved in connecting of the daughter centriole to the mother from S phase during G2. This is very interesting since proteins with such a function have not been identified. However, the manuscript does not characterize how CEP57 and CEP57L1 are achieving this linkage. This is in my opinion a slight disadvantage of the paper. Instead, the authors focus on the consequences of premature centriole disengagement in interphase. They convincingly show that centriole disengagement in G2 trigger PLK1-dependent maturation of the daughter and in addition, centriole duplication. As a consequence of this, cells enter mitosis with more than the usual (two) active centrosomes and form multi-polar spindles leading to chromosome misalignment.

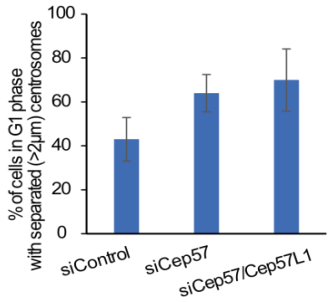
Considering that this manuscript identifies the first molecules involved in centriole interphase centriole cohesion, I strongly believe that it is suitable for publication in J Cell Biol. I have listed a number of specific points that the authors should address.

> We thank this reviewer for the supportive comment on our manuscript.

Specific points

1. Fig. 1C-E: Double depletion of CEP57 and CEP57L1 causes centriole disengagement and triggers centriole amplification. Fig. 1C shows two centriole pairs: two are close together, two are well separated. I assume that the pairs represent mother and daughter. Why are the two pairs not kept together by the centrosome linker?

> This reviewer raised a question about the centrosome linker in Cep57/Cep57L1-depleted cells. To examine whether the mother centrioles are connected by the centrosome linker, we measured the distance between two centrosomes in the G1 phase of control and Cep57/Cep57L1-depleted HeLa cells. Even in untreated HeLa cells (control), the distance was >2 µm in about 40 % of the cells, indicating that the centrosome linker did not well function in HeLa cells. In Cep57- or Cep57/Cep57L1-depleted cells, the centrosomes were a bit more frequently apart from each other (**new data is attached below**). We assume that this may be because the daughter and mother centrioles had been too apart to form the centrosome linker at the mitotic exit, due to the precocious centriole disengagement in the previous cell cycle. Considering these observations, we conclude that Cep57 and Cep57L1 are unlikely involved in the centrosome linker formation. This point is now mentioned in the figure legend of the revised manuscript (Fig. 1C).



Histograms represent frequency of the G1 phase cells with two centrosomes more than 2 µm apart. Values are mean percentages ± s.d. from two independent experiments (n = 50 for each experiment).

Why is the separation of mother and daughter relative moderate (just above 0.75 µm - the centrioles in siControl in Fig. 1C have nearly the same distance. However, I assume that these are the two mother centrioles kept together by the linker).

> Live-cell imaging of Cep57/Cep57L1-depleted cells revealed that the disengaged four centrioles were not always close together, but instead repeatedly assembled and dispersed in interphase (**new data in Video 2**). Even in the absence of the centrosomal linker, Hata et al. (2019) showed that KIFC3, a minus-end directed kinesin, generates a force to bring two centrosomes closer. Therefore, we suggest that the representative picture in Fig. 1C shows the moment when the four disengaged centrioles were assembled possibly by the force

generated by KIFC3. This point is also mentioned in the text of the revised manuscript (line 163 in page 6).

The phenotype is with 16% relatively moderate. Is this explained by the poor depletion efficiency of the two proteins? What is the depletion efficiency and does it correlate with the phenotype - please quantify (a single picture in Fig. 5C is not sufficient).

> We thank this reviewer for pointing this out. To test the depletion efficiency in Cep57/Cep57L1 co-depleted cells, we measured the expression levels in the cytoplasm and signal intensities of Cep57 and Cep57L1 (**new data in Fig. S1C, S1D**). The signal intensities of Cep57 and Cep57L1 were measured exclusively at the mother centriole marked by ODF2 signal (Fig. 4).

We revealed that, in Cep57/Cep57L1-depleted cells, the expression levels of Cep57 and Cep57L1 became 14.2% and 9.2%, respectively, compared to those in control cells, and also that 6.0% and 26.4% of the Cep57 and Cep57L1 signals, respectively, remained at the old mother centriole. In such condition, the phenotypes were observed in 27.3 % of the cells treated with siCep57/Cep57L1 for 48 h, including the cells with centriole disengagement (16%) and >4 centrioles due to centriole disengagement and reduplication (11.3%) (Fig. 1E and F). We believe that 27.3 % is a reasonably strong phenotype. The cells with >4 centrioles were frequently observed only in Cep57/Cep57L1-depleted cells and, in those cases, centriole disengagement was mostly detectable (e.g. Fig. 1C). We noticed that the treatment of siRNAs induced the phenotypes more frequently in the G2 phase (46.7%, Fig. 2). Therefore, the counting of the phenotypes in all interphase cells, including G1 phase cells with only two centrioles, contributed to the moderate phenotypes in Figure 1. We also tried to evaluate individual cells by monitoring the strength of the phenotypes and depletion efficiency. However, because simultaneously measuring the signal intensities of Cep57 and Cep57L1 at the old mother centriole requires four-color immunostaining of Cep57, Cep57L1, a centriole marker, and an old mother centriole marker, it was technically very difficult to manage. These points are now modified in the revised manuscript (line 119-124 in page 5).

2. In Fig. 1C centriole pairs stay together upon co-depletion of CEP57 and CEP57L1. This picture is different to Fig. 3C (middle). The 4 centrioles are well separated. What is the difference?

> As mentioned above, in Cep57/Cep57L1-depleted cells, the disengaged four centrioles were repeatedly assembled and dispersed (**new data in Video 2**). The immunofluorescent images of the fixed cells in Fig.1C and 3C indicate the moments of this repetition.

3. It might be worth to study Sas-6 localisation of disengaged CEP57 and CEP57L1 depleted centrioles in S/G2 phase.

> We thank this reviewer for suggesting this experiment. Following this suggestion, we counted the HsSas6 localization at disengaged Cep57 and Cep57L1 depleted centrioles in the S phase when HsSas6 is normally localized at centrosomes in control cells (**new data in Fig. S4G and S4H**). As expected, we found that HsSas6 was absent from the disengaged centrioles in Cep57/Cep57L1-depleted cells in the S phase. Importantly, in the lovastatin-arrested G1 cells in which ~30% of the centrosomes recruit HsSAS6 in control cells, the depletion of Cep57/Cep57L1 did not affect the recruitment of HsSAS6 to centrioles (**new data in Fig. S4I**). Therefore, we propose that, in Cep57/Cep57L1-depleted cells, HsSAS6 is normally recruited to the centriole in the G1/S phase for daughter centriole formation, and thereafter disappears from centrioles simultaneously with the precocious centriole disengagement. This point is now explicitly described in the revised manuscript (lane 322-331 in page 12).

4. The authors should show the mitotic localisation of CEP57 and CEP57L1. The single telophase picture in Fig. 5A suggests that CEP57L1 is not or much less associated with mitotic centrosomes. This may explain why single depletion of CEP57 in mitosis is sufficient to trigger centriole disengagement (or the mechanism is via PCNT).

> Prompted by this reviewer's comment, we compared the expression levels of Cep57 and Cep57L1 at centrosomes in interphase and mitosis. Interestingly, as this reviewer suggested, we found that Cep57 was more associated with mitotic centrioles whereas Cep57L1 was much less associated with them (**new data in Fig. S3 A-D**). So, the decrease of Cep57L1 expression in mitosis might explain why single depletion of Cep57 induces centriole disengagement during mitosis. To test this possibility, we examined whether the phenotype of Cep57 depletion was rescued by overexpression of Cep57L1. However, we found this was not the case, indicating that the specific function of Cep57 in mitotic centriole engagement cannot be explained simply by Cep57L1 reduction in mitosis (**new data in Fig. S3, H and I**). Alternatively, as the inactivation of Plk1 blocks the phenotype of Cep57

depletion in mitosis, the reorganization of PCM by Plk1 at the mitotic entry may switch the requirement of centriole engagement from the redundant Cep57/Cep57L1 mode to Cep57 mode. This point is now more explicitly discussed in the revised manuscript (lane 395-404 in page 14).

5. The authors should improve the discussion. The authors should discuss the molecular mechanism of centriole disengagement and the targets of PLK1.

> We thank this reviewer for pointing this out. We added more description to improve the discussion part in the revised manuscript. Also, we now discuss this point specifically in the revised manuscript as below.

Considering these results, we suggest that Plk1 induces the mode shift of centriole engagement from the Cep57/Cep57L1-dependent mode to Cep57-PCNT-dependent mode at the mitotic entry. However, the critical substrates of Plk1 in this process have not been determined. Given that the functional partner of Cep57 in centriole engagement changes from Cep57L1 to PCNT, we speculate that Cep57, Cep57L1 and PCNT may be critical Plk1 substrates, and that phosphorylation of these proteins by Plk1 may alter the organization of these proteins to increase the dependency of Cep57-PCNT module in mitosis. Unraveling the critical target of Plk1 in triggering this mode shift of centriole engagement will be a fascinating topic for future study. (lane 418-425 in page 15)

Why do cells have two proteins that function redundantly in this process?

> We now discuss this point in the revised manuscript as below.

Thus, on the basis of these observations, it is conceivable that the tight control of maintenance of centriole engagement is more important in interphase than in mitosis, and also that this might be a reason for the redundancy of Cep57 and Cep57L1 in the interphase centriole engagement. (lane 453-455 in page 16)

How is the daughter centriole formed in the absence of CEP152, a PLK4 adaptor.

> In the original manuscript, we measured the signal intensity of Cep152 at the mother centriole in cells treated with siCep57/Cep57L1 for 48 h, and found the significant reduction

of centrosomal Cep152 signal (Fig. 6). However, although Cep152 was reported to be necessary for centriole duplication, the reduction of centriole number was not clearly detected in Cep57/Cep57L1-depleted cells. This led us to hypothesize that the reduction of Cep152 in Cep57/Cep57L1-depleted cells was not enough to suppress the centriole duplication, at least in our experimental condition.

To examine this hypothesis, we depleted Cep152 for 48 h using two siRNAs targeting distinct sequences of Cep152 and counted the centriole number in mitosis. Although the depletion of Cep152 slightly suppressed the centriole duplication, over 70 % of Cep152-depleted cells possessed four centrioles in mitosis as control cells did, in contrast to the cells treated with siHsSASS6 (siControl: 86.7 ± 3.3%, siCep152#1: 74.4 ± 10.2%, siCep152#2: 75.8 ± 2.2%, siHsSAS6: 2.2 ± 3.8%, **new data in Fig. S4E**). The similar results were reported by Hatch et al. (2010) and Sonnen et al. (2013): Hatch et al. in Fig. 4 showed that 48 h treatment of siCep152 caused a reduction of centriole number in only 20 % of the mitotic cells, and Sonnen et al. in Fig. 6 indicated that about 70 % of the interphase cells treated with siCep152 for 72 h possessed >=2 centrioles as normal cells, and also that co-depletion of Cep152 and Cep192 caused a drastic reduction of centriole number. These observations support the notion that Cep152 and Cep192 redundantly regulate centriole duplication. Moreover, considering that the centrosomal signal of Cep152 was more effectively reduced by siCep152 than by siCep57/Cep57L1 (**new data in Fig. S4F**), we assume that the reduction of Cep152 in Cep57/Cep57L1-depleted cells was not enough to cause a defect in centriole duplication. This issue is now discussed in the revised manuscript (lane 426-445 in page 15).

CEP57 may have several functions: In interphase together with CEP57L1 it ensures centriole engagement. In mitosis it interacts with PACT of PCNT and via PCNT keeps the two centrosomes together although with an increase in distance.

> We now discuss this point in the revised manuscript as mentioned above. (lane 418-425 in page 15)

6. Fig. 9: The centriole disengagement picture shows a line connecting mother with daughter centrioles. I assume that this is PCNT. Readers may misunderstand it as the centrosome linker.

> We apologize for the confusing picture. The line was deleted in the current figure.

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

The manuscript by Ito et al reported the redundant function of cep57 and cep57L in centrosome biogenesis. The same pair of genes have been examined previously by two other groups, both of which showed that cep57/57L is required for canonical centriole duplication through recruiting an essential duplication factor cep152 to the mother centriole. Here, Ito et al uncovered a novel phenotype associated with loss of cep57/57L, which is in stark contrast to what was reported before. Ito et al did not see centriole duplication failure upon knockdown of cep57/57L; instead, they saw relatively normal initiation of centriole duplication. However, in the absence of cep57/57L, Ito et al further saw that the newborn daughter centriole that normally should form a tight association (or engagement) with the mother centriole can become prematurely disengaged before mitosis, a phenotype that would unlock the block for centriole reduplication, leading to centriole/centrosome amplification and mitotic errors. As the highlight of the story, the authors conclude that cep57/57L function together to maintain centriole engagement, ensuring the block for centriole amplification. This function, however, is completely opposite to what has been reported.

The authors made little to no effort to resolve the discrepancy mentioned above, which will be a major concern for the scientific community if not addressed. With the current data, I am a bit skeptical about the new phenotype highlighted here (see the reasons a-e listed below), but there are ways to clear the doubt (see the essential experiments 1 below).

> We appreciate this reviewer for constructive comments. As detailed below, we addressed his/her major and minor concerns.

a. All 3 labs, including the one here, observed a similar requirement of cep57/57L for cep152 recruitment. As cep152 is essential for centriole duplication, centriole assembly should be affected by loss of cep57/57L, but that was not reported in this manuscript. I therefore worry that perhaps it is the incomplete phenotype, rather than null phenotypes, that was observed, which could potentially be a result from partial knockdown of cep57/57L using RNAi.

>Prompted by this reviewer's comment, we tried to generate the Cep57/Cep57L1 double knockout Hela cells in which p53 is inactivated. However, since it was very difficult to establish the Cep57/Cep57L1 double knockout clonal cell line within four months under

recent circumstances, we decided to characterize the phenotype in a mixed population by using the CRISPR-cas9 system. In the Cep57/Cep57L1 knockout cells in which the both gene loci were targeted by sgRNAs, precocious centriole disengagement and centriole reduplication in interphase were observed as was seen in cells treated with siCep57/Cep57L1 (**new data in Fig.S1 G**). As this reviewer mentioned, the knock-down experiments with siCep57/Cep57L1 were done by another group (Zhao et al. 2020, from Yan lab). Although Zhao et al. (Fig. 4) showed a decrease in HsSAS6 foci (as a procentriole marker) in the S phase in siCep57/Cep57L1 cells, and suggested the defect in centriole duplication, we did not observe a reduction in the centriole number in the Cep57/Cep57L1 knockout cells (**new data in Fig.S1H**). Given that, in our experiments, the absence of the HsSAS6 foci in S phase was also observed in siCep57/Cep57L1 cells with four disengaged centrioles (**new data in Fig. S4G and H**), we assume that Zhao et al. perhaps observed the similar cells with four disengaged centrioles and HsSAS-6 loss. In the paper of Zhao et al., strangely, the authors did not check and quantify other standard centriole markers such as centrin and CP110. Also, Wei et al. 2020 (Kyung lab) showed a similar result on HsSAS-6 loss in the cells only with siCep57, but they did not test other standard centriole markers either. Based on these observations, we assume that the results from our and their studies are consistent with respect to the HsSAS-6 loss phenotype, but the interpretation on centriole number control is different. This point is now discussed in the revised manuscript (lane 331-335 in page 12).

b. Consequently, it is possible that the premature centriole disengagement is also an intermediate phenotype resulted from partial knockdown of cep57/57L (see d and e for details).

> As mentioned above, we examined the phenotype of the Cep57/Cep57L1 double knockout Hela cells and confirmed the similar phenotype that was observed in siCep57/Cep57L1 cells (**new data in Fig.S1G**).

c. Aziz et al (2018) showed that there is a difference in severity of phenotype for Cep57-/- mice as opposed to Cep57T/T mice which model a mutation found in human mosaic-variegated aneuploidy syndrome. Whereas the homozygous null mutation was embryonic lethal, mice with the disease mutation lived until after birth and showed supernumary centrosomes and premature disengagement. Given this evidence it is important to fully knockout Cep57 and cep57L to clarify if/what phenotype differences there may be between partial and complete protein loss.

>We thank this reviewer for the useful information.

d. One important notion about centriole assembly and centriole engagement is that they clearly share some common molecular components. For example, cep152, plk4, STIL and Sas6 are known to form the basic scaffold (as part of the cartwheel) upon which all vertebrate centrioles are built. Cep152 is also the main component of the PCM at the mother centriole where it recruits plk4/STIL/sas6 for centriole duplication. After duplication, the cep152 that is part of the PCM of the mother centriole would become embedded in the newborn centriole as part of the cartwheel and therefore, intuitively, can provide a direct physical connection between the newborn and mother centrioles. In this sense, centriole engagement and centriole duplication are functionally coupled, at least in part through cep152. It is thus entirely possible to create a situation where there is enough cep152 to drive initial centriole assembly but not enough to fully occupy the PCM for maintaining stable engagement, leading to premature detachment of newborn centrioles from mother centrioles.

> We agree with this reviewer that centriole engagement and centriole assembly could be coupled, and some common molecules could be shared in the sequential processes. One of such candidates would be Cep152 as this reviewer mentioned. To examine the extent of dependency of Cep152 on centriole duplication and centriole engagement in human cells, we depleted Cep152 for 48 h using two siRNAs targeting distinct sequences of Cep152, and counted the centriole number in mitosis (**new data in Fig. S4E**). The efficiency of Cep152 reduction at centrioles was better than that by Cep57/Cep57L depletion (**new data in Fig. S4F**). Although the depletion of Cep152 with siCep152 slightly suppressed the centriole duplication, over 70 % of Cep152-depleted cells still possessed four centrioles in mitosis as control cells did, in contrast to the cells treated with siHsSASS6 (siControl: 86.7 ± 3.3%, siCep152#1: 74.4 ± 10.2%, siCep152#2: 75.8 ± 2.2%, siHsSAS6: 2.2 ± 3.8%, **new data in Fig. S4E**). In addition, we did not observe any trend of precocious centriole disengagement in the cells treated with siCep152. The similar results were reported by Hatch et al. (2010) and Sonnen et al. (2013): Hatch et al. in Fig. 4 showed that 48 h treatment of siCep152 caused a reduction of centriole number in only 20 % of the mitotic cells, and Sonnen et al. in Fig. 6 indicated that about 70 % of the interphase cells treated with siCep152 for 72 h possessed >=2 centrioles as normal cells, and also that co-depletion of Cep152 and Cep192 caused a more drastic reduction of centriole number. These observations support the notion that Cep152 and Cep192 redundantly regulate centriole duplication. Moreover,

considering that the centrosomal signal of Cep152 was more effectively reduced by siCep152 than by siCep57/Cep57L1 (**new data in Fig. S4F**), we assume that the reduction of Cep152 in Cep57/Cep57L1-depleted cells was not enough to block centriole duplication. The same would be also true for the Cep57/Cep57L double knockout cells, because centriole duplication was not affected in these cells (**new data in Fig. S1H**). We also assume that the precocious centriole disengagement induced by Cep57/Cep57L double depletion was not simply due to the reduction of Cep152 because the efficient depletion of Cep152 with siCep152 could not induce the similar phenotype. These issues are now discussed in the revised manuscript (lane 426-445 in page 15).

e. Based on all these concerns mentioned above, the authors need to conduct essential experiments to confirm that the premature centriole disengagement seen in their assay is NOT due to partial reduction of cep152 or partial knockdown of cep57/57L. That is, I worry that when cep57/57L is fully depleted, a null phenotype where no cep152 is recruited and no canonical duplication can occur may become dominant. If so, the stated conclusion would be incorrect.

> In the original manuscript, we measured the signal intensity of Cep152 at the mother centriole in cells treated with siCep57/Cep57L1 for 48 h and found a significant reduction of centriolar Cep152 signal (Fig. 6). However, although Cep152 has been reported to be necessary for centriole duplication, the reduction of centriole number was not detected in Cep57/Cep57L1-depleted cells (Fig. 2B). To test whether the partial knockdown of Cep57/Cep57L1 masks a defect in centriole duplication, we generated Cep57/Cep57L1-knockout cells in a mixed population by CRISPR-cas9 system. However, no significant reduction in centriole number was observed (**new data in Fig. S1H**) in those cells, which led us to hypothesize that the reduction of Cep152 caused by Cep57/Cep57L1 depletion was not enough to suppress the centriole duplication, at least in our experimental condition. In contrast, we reproducibly observed the similar phenotype of precocious centriole disengagement in the Cep57/Cep57L1-knockout cells. This point is mentioned in the revised manuscript (lane 426-445 in page 15).

Essential experiments:

1. The authors must generate CRISPR knockout cells (cep57ko, cep57Lko, and cep57/57L double ko) either in clonal lines or mixed population and repeat the phenotype characterization in these KO cells. It is strongly suggested that all knockout cells should be made in p53-/- background to avoid stress response evoked by centrosome loss and the

associated mitotic insults. If the double KO cells are not viable, one can still examined the phenotype before cells die using inducible CRISPR. Null or knockout analysis is now the gold standard for centrosome research. The key is to look for null phenotypes by all means.

> We appreciate this reviewer for constructive comments on our study. Prompted by this reviewer's comment, we generated the Cep57/Cep57L1 double knockout Hela cells in which p53 is inactivated, by using the CRISPR-cas9 system, and characterized the phenotype in a mixed population. In the Cep57/Cep57L1 knockout cells, precocious centriole disengagement and centriole reduplication in interphase were observed as was seen in the cells treated with siCep57/Cep57L1 (**new data in Fig.S1G**). Although Zhao et al. in Fig. 4 showed a decrease in the number of HsSAS6 foci in siCep57/Cep57L1-treated cells and proposed the defect in the centriole duplication, we did not observe the reduction in the centriole number (marked with several centriole markers) in the Cep57/Cep57L1 knockout cells (**new data in Fig.S1H**). Importantly, given that, in our experiments, the absence of the HsSAS6 foci in S phase was also observed in Cep57/Cep57L1-depleted cells with four disengaged centrioles (**new data in Fig. S4G**), we assume that Zhao et al. perhaps observed the same thing, but interpreted that this was due to centriole duplication failure. This discrepancy might stem from the fact that they did not test other standard centriole markers in the paper. These points are now discussed in the revised manuscript (lane 129-132 in page 5 and 6 , and lane328-335 in page 12).

Other issues:

Figure 1:

Panel C:

• Cep 192 is not a good marker for the chosen experiment, as it is recruited to daughter centrioles specifically in G2. Staining for this marker clearly biases the count towards interphase cells in G2, unfairly excluding S phase cells. However, the quantification in Panels E and F do not indicate counting only cells in G2 phase, but rather all of interphase.

• Authors should use a different marker or indicate they are only counting G2 cells.

> We apologize for the confusing original figure and description. This reviewer is correct in stating that Cep192 is recruited to daughter centrioles around the G2 phase in normal cycling cells (we also reported it in the paper of Tsuchiya et al., 2016). In Figure 1C, we used Cep192 as a centrosome marker and CP110 as a centriole marker. In Fig. 1C and D, we counted the number of centrosomes in all interphase cells at random. We found that >2 Cep192 foci were observed in most of Cep57/Cep57L1-depleted cells with four disengaged

centrioles already in the S phase (**new representative picture in Fig. S1B**). Furthermore, to distinguish the Cep57/Cep57L1-depleted cells in G1, S and G2 phases, we conducted the experiments in Fig.2A-C, which also indicates that the precocious centriole disengagement occurred in the S and G2 phases. Thus, the representative panels in Fig. 1C are not limited to the G2 phase. Based on these observations, we assume that the precociously disengaged daughter centrioles are Cep192-positive already in late S phase. Figure 1E and 1F are the quantification of the indicated phenotypes in all interphase cells at random, including G1, S and G2 phases. Thus, we did not count only G2 phase cells in these experiments. These points are more explicitly described in the figure legends of the revised manuscript. (Fig. 1 D-F)

Figure 2:
Panel E:
• This figure requires a y-axis label and clarification on what is being quantified.

> We apologize for the confusing picture. We added the y-axis label in the current figure.

Figure 3:
Panel A and B:
• POC5 is recruited to newborn centrioles in G2, and is thus a marker for daughter centrioles only in G2. Disengagement, while premature, still takes time to occur, so cells with fully disengaged centrioles as judged by distance are likely at the later stages of the cell cycle, e.g. G2, which explains why POC5 is present at most of these centrioles. But S-phase cells with prematurely disengaged centrioles are mostly missed by this staining.
• Authors should quantify disengagement in S and G2 phase separately, as is done in Panel D

> We apologize for the misleading representative picture which shows the cells with four disengaged POC5-positive centrioles. We intended to state that a portion of the disengaged daughter centrioles did NOT incorporate POC5, and that daughter centrioles can be dissociated from its mother centriole before structural maturation upon double-depletion of Cep57/Cep57L1, in contrast to the normal centriole disengagement after mitotic exit. In the modified figure, the representative picture shows the cells with four disengaged centrioles, only two of which (presumably mother centrioles) are positive for POC5 (**new representative picture in Fig. 3 A**). Moreover, to clarify the difference between normal and precocious centriole disengagement, we compared the recruitment of POC5 to the

disengaged daughter centrioles between in control and Cep57/Cep57L1-depleted cells. We found that all of the disengaged centrioles were POC5-positive in most of the control cells after mitotic exit (**new data in Fig. 3 A,** 90.0 ± 3.3 %). Given that this result was in contrast to the Cep57/Cep57L1-depleted cells (only 57.1 ± 4.0% of cells with precociously disengaged centrioles were all POC5-positive, in Fig. 3B), we conclude that immature daughter centrioles can be abnormally disengaged from their mother centrioles in Cep57/Cep57L1-depleted cells. These points are now modified in the revised manuscript (line 181-189 in page 7).

Figures 3 and 4:

Panel C and D (Figure 3) / Panel E-H (Figure 4):

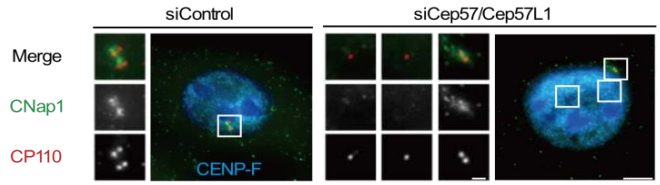
• Pericentrin is a tricky marker, as even newborn centrioles that assemble de novo will have small but clear pericentrin foci at the start of growth before acquiring MTOC competence, and will recruit significantly more as part of the PCM only after centriole-to-centrosome conversion has occurred. That is, there should not be any PCNT-negative centrioles in cells. I don't know how the authors took the images. Maybe those PCNT-negative centrioles are not real centrioles, but centrin satellites that were misjudged as centrioles. Additional markers are needed to confirm the presence of real centrioles.

> This reviewer raised the possibility that the centrin foci observed in Fig. 3 and 4 are not real centrioles, based on the assumption that there should not be any PCNT-negative centrioles. However, at least in HeLa cells which we used in the experiments, the daughter centriole is not associated with PCNT signal at the mitotic exit in control cells, when centriole disengagement occurs (**new data in Fig. S2H**). Therefore, it is possible that just after centriole disengagement, the centriole-to-centrosome conversion is not yet completed in HeLa cells.

To test the possibility that the centrin foci observed in the original manuscripts were the centriole satellite, we used CP110 as a centriole marker and repeated the experiment in Fig. 3C. (**new data in Fig. S2F and G**) As was the case for the centrin foci, only two of the disengaged CP110 foci were associated with PCNT in the S phase, and PCNT was then gradually recruited to the CP110 foci in the G2 phase and mitosis. Furthermore, the centrin signals were observed as distinct four dots in Fig. 3C, which suggests that these markers truly indicate centrioles, but not centrosomal satellites. Given these results, we conclude that daughter centrioles lack PCNT immediately after centriole disengagement in both normal and Cep57/Cep57L1-depleted HeLa cells.

• To show black and white results for MTOC competence or centrosome maturation, authors should stain for a marker such as Cnap1/cep250, but not pericentrin

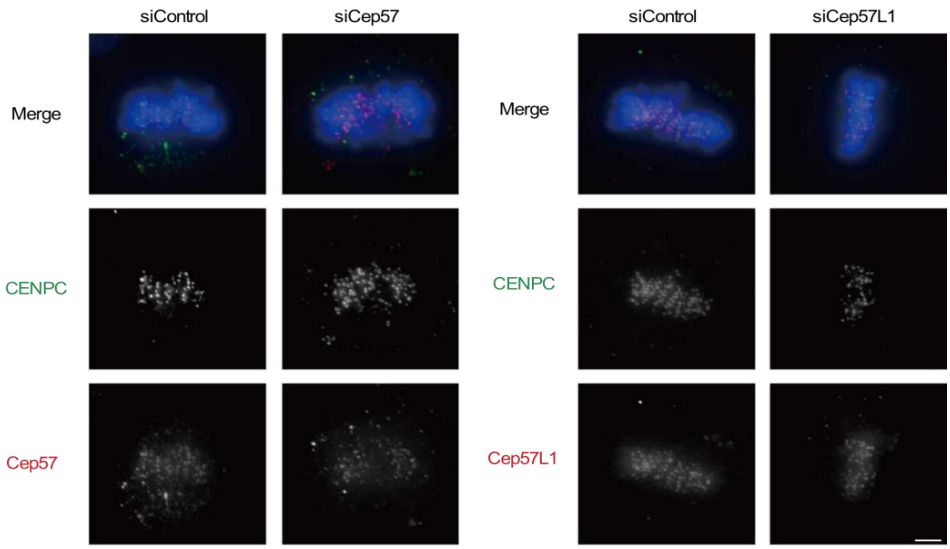
> Following this reviewer's suggestion, we immunostained Cep57/Cep57L1 depleted cells with anti-Cnap1 antibody. We found that Cnap1 was recruited to only two of the four disengaged centrioles in Cep57/Cep57L1-depleted cells as was the case for PCNT (**new data attached below**).



HeLa cells were treated with siControl or siCep57/Cep57L1, and immunostained with antibodies against CNap1 (green) and CP110 (red). Scale bar, 5 μm in the low-magnified view, 1 μm in the inset.

Figure 5:
Given the localization of Cep57 to the kinetochore as well as the centrosome it would be important for authors to examine if Cep57L1 is also present at the kinetochore.

> Prompted by this comment, we examined whether Cep57 and Cep57L1 are present at the kinetochore. Although both Cep57 and Cep57L1 signals were detected at the kinetochore by immunofluorescence with PFA fixation, the signals were not reduced by siRNA treatment. Therefore, we are not sure, at least in our experimental condition, that both proteins are associated with the kinetochore (**new data attached below**). Given that the data is not relevant to the main claims in this manuscript, we decided not to present the data.



HeLa cells were treated with siControl, siCep57 or siCep57L1, and immunostained with antibodies against CENPC (green) and Cep57/Cep57L1 (red). Scale bar, 5 μm,

Figure 6:

The diagram in Figure E does not depict the redundancy and effect of combined knockdown of both Cep57 and Cep57L1, particularly on cep152 recruitment. Perhaps authors can include an additional colored arrow to indicate effect of the combined knockdown.

> We apologize for the confusing picture. We clarified the effect of the combined knockdown of Cep57/Cep57L1 on the Cep63 and Cep152 recruitment in the current figure.

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

In this MS, Ito and colleagues describe Cep57L1 as a component that together with Cep57 maintains centrioles engaged in interphase. In contrast to Cep57, Cep57L1 is not required for centriole engagement in mitosis, implying an interphase specific function for Cep57L1. In interphase, Cep57 and Cep57L1 maintained engaged centrioles independently of Plk1. Prematurely disengaged centrioles upon Cep57/Cep57L1 co-depletion acquire pericentriolar material proteins (PCM) prior to mitosis and convert into functional centrosomes that nucleate microtubules. This leads to the formation of multi-polar spindles and chromosome mis-segregation, highlighting the importance for this regulation.

Overall, I found the study very interesting. The mechanisms that keep centrioles engaged in interphase are still poorly understood and this paper now shows that Cep57L1 and Cep57 cooperatively control this process. The experiments are of high quality and the authors provide a comprehensive analysis of Cep57/Cep57L1 depleted cells. However, I miss a clear model to explain why Cep57L1 is specifically involved in centriole engagement in interphase but not mitosis.

> We thank this reviewer for the supportive comment on our manuscript.

Specific points:
1. I found quite puzzling that Cep57 but not Cep57L1 is required for centriole engagement in mitosis. A simple explanation could be that Cep57L1 is degraded or not present at centrioles in mitosis, explaining the requirement for both proteins in interphase but only Cep57 in mitosis. This could be easily tested by analyzing Cep57L1 protein levels and centriole association at different stages of the cell cycle.

> We thank this reviewer for suggesting this experiment. Following this suggestion, we compared the expression levels of Cep57 and Cep57L1 at centrosomes in interphase and mitosis. Interestingly, as this reviewer suggested, we found that Cep57 was more associated with mitotic centrioles whereas Cep57L1 was much less associated with them (**new data in Fig. S3 A-D**). So, the decrease of Cep57L1 expression in mitosis might explain why single depletion of Cep57 induces centriole disengagement during mitosis. To test this possibility, we examined whether the phenotype of Cep57 depletion was rescued by overexpression of Cep57L1. However, we found this was not the case, indicating that the

specific function of Cep57 in mitotic centriole engagement cannot be explained simply by Cep57L1 reduction in mitosis (**new data in Fig. S3, H and I**). Alternatively, as the inactivation of Plk1 blocks the phenotype of Cep57 depletion in mitosis, the reorganization of PCM by Plk1 at the mitotic entry may switch the requirement of centriole engagement from the redundant Cep57/Cep57L1 mode to Cep57 mode. This point is now more explicitly discussed in the revised manuscript (lane 395-404 in page 14).

2. In figure 5B, the authors analyze Cep57L1 co-localization with PCNT and inferred that Cep57L1 forms a ring-like structure at the mother centriole that is similar to the ring formed by Cep57. However, this conclusion was based on previously published data for Cep57 (Watanabe et al. 2019). Ideally, the authors should co-stain Cep57 and Cep57L1 for better comparison. This should be feasible considering that rabbit and mouse antibodies are available for Cep57L1 and Cep57, respectively.

> We thank this reviewer for the constructive comment. Following this suggestion, we observed Cep57 and Cep57L1 simultaneously by STED microscopy using anti-Cep57 antibody produced in mouse and anti-Cep57L1 antibody produced in rabbit, and showed the colocalization of both proteins around a centriole (**new data in Fig. S3 E**). To confirm this data, we also quantified the radiuses of Cep57/Cep57L1 rings individually (Cep57, 108.3 ± 8.1 nm: Cep57L1, 111.1 ± 15.6 nm, **new data in Fig. 5B**).

This will also be important considering that Cep57L1 centriolar levels increase upon Cep57 depletion. Is the location of Cep57L1 rings also changed upon Cep57 depletion?

>We thank this reviewer for the suggestion. We tested the localization of Cep57 and Cep57L1 under reciprocal depletion. We found that the localization of both proteins was not significantly affected by each other, but the radius of Cep57L1 ring became a bit smaller upon Cep57 depletion, suggesting that Cep57L1 was localized closer to the mother centriole wall in the absence of Cep57. This observation is now mentioned in the revised manuscript (**new data in Fig.S3 F and G**).

Minor points

1. Please check the y-axis of the graph shown in Figure 2E. Do the authors mean cells instead of percentage of cells?

> We apologize for the confusing picture. We added the y-axis label “% of cells” in the current figure.

2. The authors should cite the recent publication showing that Cep57 and Cep57L1 recruits Cep63-Cep152 complex for centriole biogenesis (Zhao et al., JCS 2020).

> Following this comment, we cited the publication (Zhao et al., JCS 2020) in the revised manuscript.

December 7, 2020

RE: JCB Manuscript #202005153R

Prof. Daiju Kitagawa
University of Tokyo
Hongo 7-3-1
Bunkyo-ku, Tokyo 113-0033
Japan

Dear Dr. Kitagawa,

Thank you for submitting your revised manuscript entitled "Cep57 and Cep57L1 maintain centriole engagement in interphase to ensure centriole duplication cycle." We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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

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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.**

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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. You currently do not have scale bars in supplementary figure 1B.

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) Abstract and title: The summary should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces.

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7) 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.

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.

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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.

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

Sincerely,

Yixian Zheng, Ph.D.
Monitoring Editor
Journal of Cell Biology

Dan Simon, Ph.D.
Scientific Editor
Journal of Cell Biology

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

The authors have addressed all the points that I raised during my first evaluation.
I recommend publication of their manuscript in J Cell Biology.

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

The authors satisfactorily addressed my concerns. I support publication of the revised manuscript without further comments.