JCB

Phosphorylation of CEP83 by TTBK2 is necessary for cilia initiation

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Re: JCB manuscript #201811142

Prof. Won-Jing Wang
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Dear Prof. Wang,

Thank you for submitting your manuscript entitled "TTBK2 phosphorylates CEP83 in promoting cilia initiation". Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

You will see that while the reviewers feel that the manuscript is largely technically sound, more data is needed to shore up the conclusions and to rule out alternative interpretations. In particular, you should pay particular attention to the following points:
1) Demonstrating the phosphorylation of endogenous Cep83 by Ttbk2. As pointed out by reviewer#1, the model predicts that Cep83 phosphorylation will increase upon serum starvation and this should be tested. All reviewers express concerns that making a case for a kinase substrate based strictly on overexpressed proteins is not sufficient and needs to be corroborated with data on the endogenous proteins. Fig. 6A and C show that you have the tools to detect endogenous Cep83 and to detect its phosphorylation. Therefore, it seems reasonable to ask that you expand upon 6C by performing siRNA for Ttbk2 as well as performing careful quantitation of the signals. You may consider metabolic labeling with inorganic 32P to maximize signals.

2) A more complete investigation of the phenotype of the RPE Cep83-/- cells rescued by the 4A and DEED mutant is warranted. Revs#2 and #3 have excellent suggestions for pursuing this. Fig. 6B should be expanded and subjected to quantitative analysis.

We also hope that you will be able to address all of the other points raised by reviewers #2 and #3 in full. Finally, while we agree with rev#1 that examining the function of TTBK2-dependent phosphorylation of CEP164 and 89 and examining the timing and location of TTBK2-dependent phosphorylation of CEP83 are both intriguing avenues of investigation, we feel that these pursuits are somewhat beyond the scope of the current work and so we would not require them for resubmission (though, if you choose to add any of this data, you are welcome to do so).

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. Our typical timeframe for revisions is three to four months; if submitted within this timeframe, novelty will not be reassessed. We would be open to resubmission at a later date; however, please note that priority and novelty

would be reassessed.
If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count 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: Your manuscript may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.
IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Your manuscript 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.

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, 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. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.
Sincerely,

Maxence Nachury, PhD
Monitoring Editor
JCB

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

The work describes here expands on previous work from Tanos et al. to further define the hierarchy of events that controls the assembly of centriole distal appendages, structures that are required for docking of ciliary vesicles to the membranes and maturation of mother centriole in order to build a

primary cilium. In this manuscript, they incorporate TTBK2, a kinase required for cilia initiation, into the distal appendage pathway. It was previously shown that the distal appendage protein CEP164 is required for recruitment of TTBK2Cto the mother centriole, and it was known that CEP164 recruitment to the mother centriole depends on the presence of CEP83. Here they argue that phosphorylation of CEP83 at the mother centriole by TTBK2 is important for cilia docking and maturation. These are interesting findings, but the experiments also raise a number of questions, listed below.

No evidence of direct interaction between CEP83 and TTBK2 is provided. Although the authors show by co-IP that the C-terminal domain of TTBK2 is required for their interaction with CEP83, DAP proteins form a complex and the C-terminal domain of TTBK2 was previously shown to bind CEP164. To rule out an indirect interaction, additional experiments would be required; for example, a yeast-2-hybrid experiment or co-IP in Cep164 null cells. Similarly, TTBK2 for the in vitro kinase assays was purified from 293T cells, which could lead to copurification of interacting protein(s) that might phosphorylate CEP83. A bacterially-expressed purified TTBK2 kinase would better support their conclusion that TTBK2 phosphorylates CEP83 directly.

In addition to CEP83, the data in the manuscript shows that TTBK2 can phosphorylate two other DAPs protein, CEP89 and CEP164, raising the question of which (if any) is the main principle target of TTBK2 during cilia initiation? Can the authors identify the phosphorylation sites on CEP89 and CEP164 test whether they are important for function? It is noteworthy that in the co-expression experiment, TTBK2FL alone (without CEP83) pull-downs another phosphorylated protein similar in size similar CEP83 (Figure 3C and 3F, detected by P-Ser/Thr antibody). Hence a phosphorylated peptide analysis by Mass spectrometry of TTBK2 interaction proteins might be a better approach for systematic identification of TTBK2 substrates.

Using super-resolution microscopy, Lo et al analyzed the recruitment and redistribution of TTBK2 from periphery toward the root of mother centriole upon serum starvation. However this was shown in a paper from the same group early this year (Yang et al., 2018, figure 4) and the basic observation was made in Goetz et al., 2012.

An important, but challenging question is where and when does phosphorylation of CEP83 take place in the cell. The authors only identified the four peptides phosphorylated when TTBK2 is over-expressed but not in the control samples, which indicates the phosphorylation level of CEP83 in vivo is low. What is the phosphorylation state of CEP83 in resting and dividing cells? Ideally, an antibody that recognizes phospho-CEP83 could answer these and related questions.

The phosphorylation-deficient mutant form CEP834A still allows cilia to form at reduced frequency (25% of cells are ciliated). To account for the deficit in cilia formation in the CEP834A mutant, the authors example two processes important in cilia initiation: removal of CP110 and association with ciliary vesicles. Both process are reduced, but not abolished, in the mutant. Together with the identification of other possible TTBK2 phosphorylation sites, this suggests some overlap in function between TTBK2 targets. This would be very interesting to explore.

Minor concerns:

(1) Fig 5B WB result shows the expression of WT CEP83 protein in Cep83 ko cells. However, in the blot of CEP83 panel there appears to be endogenous CEP83 protein band (lower band in the top

blot), which should be absent in CEP83 ko cells.

(2) In the Fig 2B phos-tag gel blot, co-expressed TTBK2FL induced a phosphorylation-dependent shift in about 50% of CEP83 protein to be shifted (phosphorylated), while in the similar experiment in C, 100% is shifted. What is the basis of this difference?

(3) Figure 1A, use "pGlu-Tub" instead of "Glu-Tub" to label Polyglutamylation tubulin. Detyrosinated tubulin also designated as Glu-Tub.

(4) Fig 2D, F. The molecular weights of CEP83 and 89 are not consistent.

(5) Figure 5K middle panel, the zoom in cropped box is shifted.

(6) There is no antibody information on Myo-Va in the Supplementary Table 2.

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

In this paper, the authors found CEP83 as a novel substrate of TTBK2. The general quality of the data is high, and I especially appreciate that the authors performed rescue experiments for key data. I am convinced that CEP83 is probably a bona fide target of TTBK2. But the weak point of this paper is that any biological role of this phosphorylation is very unclear, and it has to be addressed before being appropriate for publication.

Major point
(1) In figure 6D, authors created a phosphorylation-defective mutant (4A) and showed that the 4A mutant partially failed to rescue a ciliation defect of CEP83 knockout cells. However, a possible explanation for the failure is that the substitutions from serine/threonine to alanine might partially disrupt the structure of CEP83, and thus results in partial dysfunction of the protein. Also, the phospho-mimic mutant (DEED) in this figure added little to the conclusion.
To strengthen the conclusion, it would be valuable to perform a rescue experiment with the phospho-mimic mutant (DEED) in proliferating cells. It would greatly strengthen the conclusion, if the phospho-mimic mutant is sufficient for removing CP110 and/or recruiting ciliary vesicle, thus for inducing cilium formation.

(2) The authors conclude that phosphorylation of CEP83 is important for CP110 removal and ciliary vesicle docking. I think the most likely possibility is that the phosphorylated CEP83 regulates those steps via other distal appendage proteins because :

i) Based on classical electron microscopy studies, ciliary vesicle is recruited to the middle-distal region of the distal appendage, whereas CEP83 localizes to the proximal region of the distal appendage.
ii) A distal appendage protein, CEP164, regulates both CP110 removal and ciliary vesicle docking. Further, CP110 removal might be a downstream of ciliary vesicle docking, as shown in the previous paper (https://www.ncbi.nlm.nih.gov/pubmed/25686250).
iii) CEP83 is required for the localization of other distal appendage proteins.

In that sense, it would be valuable to test whether the localization of other distal appendage proteins is affected in the CEP83 knockout cells expressing phosphorylation defective (4A)-CEP83. The authors might have addressed this in the Fig. 6B, but some quantification is needed. All other distal appendage proteins, including LRRC45, should be tested. Moreover, authors should test the localization of IFT-B and transition zone components, as their localization is dependent on CEP164 http://jcb.rupress.org/content/199/7/1083.

Minor point
(1) Page 3, line 6. Although authors mentioned LRRC45 in the discussion, LRRC45 should be mentioned in the introduction as well. Is TTBK2 not a distal appendage protein?

(2) Page 3, line 7. In this context, authors should refer the papers that discovered the distal appendage proteins (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2064767/ and https://www.ncbi.nlm.nih.gov/pubmed/21976302) rather than the papers that have listed distal appendage proteins on them, specifically (Lu et al., 2015) and (Schmidt et al., 2012).

(3) page 3, line 3. MMP9 paper should be mentioned not only in the discussion but also in the introduction.

(4) page 5 lines 1-3 and page 11 lines 2-4. The authors should discuss a potential mechanism for the TTBK2 redistribution. Given that CEP164 is absolutely required for TTBK2 localization, CEP164 distribution might also change upon serum deprivation. The distribution pattern shown looks highly similar to that of CEP164 (Fig. S2 of Yang et al., 2018). It is not clear if this CEP164 image was taken under serum-starved condition, but if this is the case, it would be very interesting to see the localization of CEP164 in proliferating cells.
Another possibility is that two peaks (150 nm and 225 nm) of CEP83 radial diameter cannot be observed in proliferating cells, because of the weaker signal (Fig. 1C, right graph and Fig. 1E).

(5) page 12 line 24 and page 13 line 1. "nephronophthisis (NHPH18)" should be "nephronophthisis (NPHP18)"

(6) page 13. Authors should greatly improve their methods section to ensure reproducibility. Much important information are missing.

(A) Methods for "purification of recombinant protein", "co-immunoprecipitation", "quantification of signal intensity" are missing.

(B) page 13 line 13. "complete medium". Which media? Did authors use the same media for all cell lines?

(C) page 13 line 14. "transient transfections" What is the method for a transient transfection? How many cells were plated into which dish? How much DNA and transfection reagent were used?

(D) How did authors create a retrovirus? Which cells were used? How much of puromycin was used to select the cell lines?

(E) page 13 lines 21, 23 and 27. "pcDNA3-FH or pRK5M vector", "pBabe-puro3", "pET32a". Are there references and/or catalog numbers for these vectors? Does pcDNA3-FH vector contain

FLAG-HA tag at N-terminus or C-terminus? What was the pRK5M vector used for? (i.e. In which experiments did authors use this vector?).

(F) page 14 line 19. "incubating with the indicated primary antibodies". How long?

(G) page 14 line 19 "Secondary antibodies were all from molecular probes". Molecular probes provide a variety of secondary antibodies. Authors should specify fluorophores and the catalog number of each secondary antibody.

(H) page 14 line 23. Objective lenses (with NA) are missing.

(I) page 15 line 2. Catalog numbers for PVDF membrane are missing. Depending on the detection method (chemiluminescence or fluorescence), a specific PVDF membrane has to be chosen.

(J) page 15 line 4. Catalog numbers for alkaline phosphatase is missing. NEB provides several alkaline phosphatases. Which one?

(K) page 15 "dSTORM" Which chamber was used? How many cells were plated? How long the samples were incubated with primary/secondary antibodies? What was the concentration of primary/secondary antibodies? This information is very important because inefficient staining could result in the defect of a super-resolved structure (https://www.ncbi.nlm.nih.gov/pubmed/22735543)
Which fluorophores were used? Alexa647 for single color? And Alexa647 and Cy3B for 2 color?
Which laser lines? Was the activation laser used? How many frames were typically recorded? What is the pixel size of the final image?
How did author calculate the radial diameters?

(L) page 15 line 23 "Tetraspeck" What diameter? Please specify the catalog number.

(M) page 15 line 25. What are the concentrations of Tris-HCl and NaCl.

(N) page 16 line 2. How many 293 cells were plated in which dish? What volume of M2 beads were used? Was the kinase assay performed on the beads? Or FLAG-tagged TTBK2 was eluted with FLAG-peptide? Were the beads washed with kinase buffer before adding CEP83? How much purified CEP83 was added?

(O) page 16 line 9. Authors should also include how the phosphorylation was detected. (Added sample buffer, performed SDS-PAGE etc...).

(7) page 25 line 9. "FLAG-tagged CEP83" This seems like HA-tagged CEP83, otherwise subsequent detection with HA (Fig. 4D) does not make sense.

(8) page 26 line 2. "Cells were serum starved to induce cilia formation" How long?

(9) page 26 line 14. "G1-arrested cells" How long were the cells serum-starved?

(10) Figure 2A and Figure 5B. Authors detected FBF1 at 75 kDa (Fig.2A) and 55 kDa (Fig. 5B). Please confirm that authors actually detected FBF1, because the molecular weight of the main isoform of this protein is 125 kDa. The previous paper showed FBF1 protein around 130 kDa. https://www.nature.com/articles/ncomms3750

(11) Supplementary table 2. "anti-centrin 2" Authors seemed to use centrin antibody, but not centrin 2 antibody. The catalog number indicates centrin antibody.

The anti-Myosin Va antibody is missing.

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

In the manuscript entitled "TTBK2 phosphorylates CEP83 in promoting cilia initiation", the authors present compelling evidence that the kinase activity of TTBK2 promote ciliogenesis in phosphorylating CEP83 at centriole distal appendages. The study is comprehensive, starting with the observation that TTBK2 redistributes its localization at centriole distal appendages closes to CEP83 during ciliogenesis. The authors then identify CEP83 as a novel substrate for TTBK2 and characterize four phosphorylation sites. They show that TTBK2-dependent CEP83 phosphorylation is required for the initial stages of ciliogenesis in RPE1 cells, in particular the formation of the ciliary vesicle and the removal of CP110 from the centriole distal end. The authors also mapped the domain in CEP83 required for targeting of the distal appendage components and recruitment of TTBK2 to the mother centriole.
Overall the manuscript is well written and quite informative. This is a significant finding regarding cilia initiation mechanism involving TTBK2 via its kinase activity in phosphorylating DAPs at the centriole. The methods used (STORM, SIM, Mass spectrometry and CRISPR-Cas RPE1 cell lines) are state of the art and the results support the conclusions made.
However, my main concern is the absence of quantification of Western blot analyses (with statistical analysis) and of fluorescence intensity of the proteins recruited at the centrioles presented throughout the manuscript (Fig. 2A, F, 3A, D-G, 6C and Fig. 5C-J, 6B, C respectively).
Specific points:
1- Fig.2A: Quantification of WB analysis of centriolar proteins is required, in particular for the bands corresponding to CEP164 and FBF1 that seem slightly increased in serum starved conditions.
2- Fig. 2B-Fig. S1: The authors observe that " CEP83 showed an upward gel mobility shift on reducing gel and phospho-tag gel when full-length TTBK2". Is the upward gel mobility shift specific to CEP83? Have other DAP components been tested? Why biochemistry has been performed on overexpressed proteins only? What about the endogenous CEP83?
3- Fig. 2D: The meaning "FH" as Flag tagged should be indicated in the Figure legend or in the text. Why the authors used so many different constructs in not clear.
4- Figure S2A. The authors claim that the interaction between CEP83 and TTBK2 "was not relied on the TTBK2 kinase activity, as expression of TTBK2FL and TTBK2KD showed no difference for their interaction." Quantification of several experiments is required as CEP83Myc seems to bind less TTBK2KD than TTBK2FL and especially as the amount of TTBK2FL and TTBK2KD precipitated is not equivalent.
5- The authors do not provide evidence of interaction/phosphorylation with the endogenous proteins CEP83 and TTBK2. Why not observe the phosphorylation status of the endogenous CEP83? What about the endogenous interaction? Overexpressed cytosolic proteins in cells expressing the endogenous protein might not be engaged in the interaction. What about the localization and co-localization of TTBK2KD and CEP83 in those cells?
6-The Fig.3D-G lacks quantification. Fig. 3G: the annotation of the "Flag-TTBK2" is missing on the figure legend. Moreover, image corresponding to the Commassie blue coloration lacks annotations: molecular weight marker is missing, CEP83-HIS... What is the size of the HIS-CEP83 products?

According to the Fig. 3G corresponding band is above 100 kDa. Why is it so different from the size of Flag-CEP83 that migrates above 75 kDa in the other Figures?
7- Fig.4C: The authors generate RPE1 cell line (wt or SCKT1 KO) stably expressing CEP83-Flag. Same remark as before, why not look at the endogenous CEP83 protein in those cells?
8-Fig.S4A: Quantification should be provide.
9- Fig.5B "Flag and HA-tagged CEP83 was stably expressed in CEP83-/- cells. Western blot analysis was performed with antibodies as indicated." An additional band is detected by the CEP83 antibodies in CEP83-/- cells stably reexpressing CEP83-Flag, which that is not recognized by the Flag antibodies and that may correspond to the endogenous CEP83 protein. It may also be a cleavage/degradation product of the reexpressing CEP83 protein lacking the Flag tag. Same remark for the WB presented in Fig. S6. What about the mRNA expression in CEP83-/- cells? If the Cep83 transcript expression is down in those cells it can be an easier way to confirm the absence of endogenous CEP83.
10- Fig. 5F: The authors use U2OS cells to map the distal appendage-targeting domain in CEP83? Why the use of this additional cell line? This can be confusing to multiply the number of cell lines.
11- Figures 5H and 5I: WB of stable RPE1 cells reexpressing the CEP83 mutants (151E, 367E) should be provide.
12- A quantification of the recruitment of CEP164 and CEP89 (Figures 5H and 5I) and CEP83, CEP164, TTBK2 (Fig. 6B) at the DAP should be provide. Of note, in Fig. 5I, CEP164 inset is different from the CEP164 staining in the merged picture. Most probably a duplication of the HA staining. As a decrease of CEP164 or CEP89 may altered the integrity of the 9 doublets of centriolar distal appendages, STORM analyses could be helpful to confirm their integrity.
13- Fig. 6C: The quality of the blot is poor. Quantification of independent experiment should be provided.
14- In the discussion part, the authors do not discuss how they reconcile the recruitment of TTBK2 by CEP164 at the DAP and the TTBK2 redistribution from the periphery toward the root of centriole distal appendage close to CEP83 to allow it's phosphorylation"?
Minor changes:
1- Fig4D: "CEP83-Flag" instead of "CEP83-HA".
2- Fig.5A: Page 8 line 1, "... a clone of T deletion in both alleles,..." should be rephrasing as "...a clone with a one base pair (T) deletion on both alleles"
3- Page 11 line 29; MPP9 is misspelled
4- Page 12 and 13: NPHP18 is misspelled

Dear Maxence and Tim,

We would like to thank the reviewers and editors for the positive notes and critical suggestions. We have revised the manuscript according to the comments. Significant changes are itemized below, followed by our point-by-point responses that address reviewers' concerns.

Sincerely,
Won-Jing

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NEW ITEMS:

1. Figure 1F-J, Figure 2K, and Figure 3F-J: We generated *TTBK2-/-* RPE1 cells (Fig. 1F). By comparing with wild-type RPE1 cells, serum starvation-induced CEP83 phosphorylation was not observed in *TTBK2-/-* cells (Figs. 3F, 3I, and 3J). The data show that CEP83 phosphorylation is mediated by TTBK2. We also used *TTBK2-/-* cells to examine whether the redistribution of TTBK2 at the centrioles depended on TTBK2's activity by re-expressing full-length ($TTBK2^{FL}$) and kinase dead of TTBK2 ($TTBK2^{KD}$) in *TTBK2-/-* cells (Figs. 1G and 1H). Both $TTBK^{FL}$ and $TTBK2^{KD}$ formed smaller rings at the centrioles and co-localized with CEP83 upon serum starvation. The results indicate the redistribution of TTBK2 at the centrioles upon serum starvation does not rely on the kinase activity of TTBK2 (Figs. 1I, 1J, and 2K).

2. Figure 3G-J, Figure 4 I&J, and Figure S2 D-F: We generated two phospho-CEP83 antibodies (anti-phospho-$CEP83^{S29}$ and anti-phospho-$CEP83^{T292}$; Figs. S2D-S2F) to examine whether endogenous CEP83 phosphorylation occurred during ciliogenesis. We also used the phospho-CEP83 antibodies to examine whether the serum starvation-induced phosphorylation of endogenous CEP83 was mediated by TTBK2.

We performed immunostaining with the phospho-CEP83 antibodies in unsynchronized cells. Our results showed that the phosphorylated $CEP83^{S29}$ and $CEP83^{T292}$ signals at the centrioles were higher in ciliated cells than in non-ciliated cells, indicating CEP83 phosphorylation was increased during ciliogenesis (Figs. 3G and 3H). In addition, we used the antibodies to analyze endogenous CEP83 phosphorylation in wild-type and *TTBK2-/-* cells. We found that the percentage of cells with positively phospho-

CEP83^{S29} and phospho-CEP83^{T292} signals increased during serum starvation in wild-type cells and this serum starvation-induced CEP83 phosphorylation was not seen in *TTBK2-/-* cells (Figs. 3I and 3J). Together, our results clearly indicate that endogenous CEP83 phosphorylation (Ser29 and Thr292) is induced during ciliogenesis and is mediated by TTBK2.

We also used the phospho-CEP83 antibodies to examine whether the recruitment of TTBK2 by CEP164 affected TTBK2's role in phosphorylating endogenous CEP83. In *SCLT1-/-* cells, CEP83 phosphorylation was not induced by serum starvation, suggesting that the recruitment of TTBK2 to centriole distal appendages by CEP164 is required for TTBK2 to phosphorylate CEP83 (Figs. 4I and 4J).

3. Figure S2: We performed 2-D gel electrophoresis in wild-type and *TTBK2-/-* cells to analyze the overall change of endogenous CEP83 phosphorylation. Cells were serum starved for 2 days to induced ciliogenesis. In serum-starved cells, CEP83 was resolved by its charge into two main spots that were consistent with the theoretical isoelectric point and molecular weight of CEP83. We found there was a right-shifting spot in the *TTBK2-/-* cell lysate or in cells treated with phosphatase prior to gel analysis (Fig. S2C), indicating CEP83 phosphorylation was mediated by TTBK2.

4. Figure 6, 7, and Figure S5: We performed a more complete analysis of the phenotypes in *CEP83-/-* RPE1 cells that re-expressed CEP83 phospho-inactive or phospho-active mutants (CEP83^{4A} or CEP83^{DEED}). Key steps during ciliogenesis were analyzed in proliferating and G1-arrested cells to examine the influence of CEP83 phosphorylation during ciliogenesis.

First, we found that the ciliated frequency of CEP83^{DEED} expressing cells was higher than CEP83^{WT} and CEP83^{4A} expressing cells in proliferating cells (Figure 6D). Further supporting our conclusion that CEP83 phosphorylation promotes ciliogenesis.

To test whether CEP83 phosphorylation affected distal appendage assembly and structure, we quantified fluorescent intensities of various distal appendage proteins (CEP164, CEP89, FBF1, SCLT1, and LRRC45) in proliferating and G1-arrested cells. The results showed no difference in CEP83^{WT}, CEP83^{4A}, and CEP83^{DEED} expressing cells, indicating that CEP83 phosphorylation didn't affect the recruitment and structure of centriole distal appendages (Figs. 6E&6F and Figure S5D).

To test whether CEP83 phosphorylation affected the recruitment of proteins to the centrioles via centriole distal appendages, we quantified the fluorescent intensities of TTBK2 and IFT88. The fluorescent intensities of TTBK2 and IFT88 showed no difference in CEP83^{WT}, CEP83^{4A}, and CEP83^{DEED} expressing cells, indicating that CEP83 phosphorylation didn't affect the recruitment of TTBK2 and IFT88 (Figs. 7A and 7B).

To further confirm the effect of CEP83 phosphorylation on CP110 removal and

ciliary vesicle docking, we also performed the staining of CP110 and myosin-Va in proliferating cells. The CEP83^{DEED} expressing cells showed more CP110 was removed from the mother centrioles and contained more ciliary vesicles when compared with the CEP83^{WT} and CEP83^{4A} expressing cells (Figs. 7F and 7G).

Since the removal of CP110 from mother centrioles and the docking of membrane vesicles to the mother centrioles promote transition zone assembly, we also analyzed the effect of CEP83 phosphorylation in the establishment of transition zone. Ciliogenesis was induced by serum deprivation. Our results showed that most of CEP83^{4A} expressing cells contained less TCTN2 and NPHP1 positive signals at the cilia base when compared to the CEP83^{WT} or CEP83^{DEED} expressing cells, indicating the establishment of transition zone was affected by CEP83 phosphorylation (Fig. 7E).

5. Figure 4C: We generated *CEP164-/-* 293T cells to examine whether the recruitment of TTBK2 by CEP164 at the centriole distal appendages affected TTBK2-CEP83 interaction. By Comparing with the wild-type RPE1 cells, TTBK2 didn't interact with CEP83 in the *CEP164* knockout cells, indicating that the interaction between TTBK2 and CEP83 required CEP164.

6. Methods: New details were added to each section in the materials and methods. In addition, new sections titled "**Retrovirus production and infection, Quantification of images from immunostaining and immunoblots**, **Purification of recombinant proteins, co-immunoprecipitation, Peptide competition assay, and 2-D gel electrophoresis**" were also added to the methods, describing how we produced retrovirus, purified recombinant proteins, quantified images from immunostaining and immunoblots, confirmed the specificities of phospho-CEP83 antibodies, and performing 2-D gel electrophoresis. The list of antibodies is now incorporated into the material and methods section "**primary antibodies**".

7. Other changes are detailed below in our point-by-point response.

POINT-by-POINT RESPONSES:

Reviewer #1 (Comments to the Authors (Required)):
Major point
No evidence of direct interaction between CEP83 and TTBK2 is provided. Although the authors show by co-IP that the C-terminal domain of TTBK2 is required for their interaction with CEP83, DAP proteins form a complex and the C-terminal domain of TTBK2 was previously shown to bind CEP164. To rule out an indirect interaction, additional experiments would be required; for example, a yeast-2-hybrid experiment or co-IP in Cep164 null cells. Similarly, TTBK2 for the in vitro kinase assays was purified from 293T cells, which could lead to copurification of interacting protein(s) that might phosphorylate CEP83. A bacterially-expressed purified TTBK2 kinase would better support their conclusion that TTBK2 phosphorylates CEP83 directly.

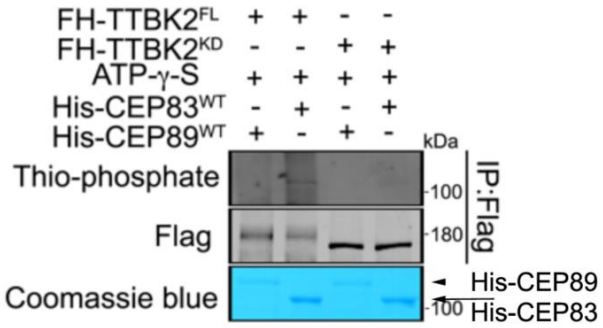
We thank the reviewers for these comments and nice suggestions. To analyze the influence f CEP164 in TTBK2-CEP83 interaction, we generated *CEP164-/-* 293T cells and performed co-IP experiments (Fig. 4C). Our result showed that CEP83 was not detected in the immunoprecipitated TTBK2 complex in *CEP164-/-* cells, indicating that CEP164 is required for TTBK2-CEP83 interaction.

To prevent the possible co-purification of other enzymes in the TTBK2 immunocomplex that might also phosphorylate CEP83, we expressed and purified TTBK2 from bacteria. By using the recombinant TTBK2 as the enzyme and recombinant CEP83 as the substrate, our result clearly show that TTBK2 directly phosphorylates CEP83 in the in vitro kinase assay (Fig. 3C).

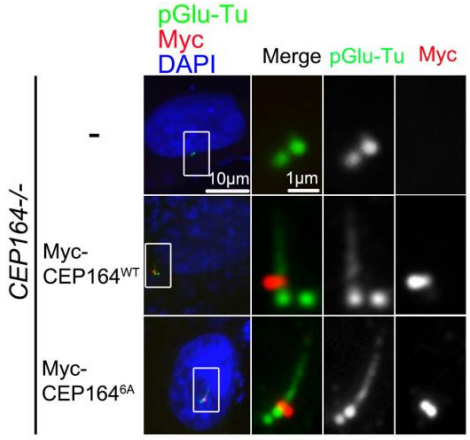
In addition to CEP83, the data in the manuscript shows that TTBK2 can phosphorylate two other DAPs protein, CEP89 and CEP164, raising the question of which (if any) is the main principle target of TTBK2 during cilia initiation? Can the authors identify the phosphorylation sites on CEP89 and CEP164 test whether they are important for function? It is noteworthy that in the co-expression experiment, TTBK2FL alone (without CEP83) pull-downs another phosphorylated protein similar in size similar CEP83 (Figure 3C and 3F, detected by P-Ser/Thr antibody). Hence a phosphorylated peptide analysis by Mass spectrometry of TTBK2 interaction proteins might be a better approach for systematic identification of TTBK2 substrates.

In our revision, we studied the roles of TTBK2-dependent CEP89 and CEP164 phosphorylation in ciliogenesis. Our results indicated that CEP89 was not a TTBK2 substrate (Fig. 2G and the figure provided below). We also mutated the candidate TTBK2 phosphorylation sites on CEP164 (CEP164^{6A}) and re-expressed CEP164^{6A} in *CEP164-/-* cells to test the role of TTBK2-dependent CEP164 phosphorylation in ciliogenesis. By comparing with CEP164^{WT} expressing cells, ciliogenesis showed no difference in CEP164^{6A} expressing cells. More experiments are required to understand the relationship between CEP83 and CEP164 phosphorylation. Detailed information regarding how we analyzed CEP89 and CEP164 phosphorylation is listed below.

1. We analyzed CEP89 phosphorylation during ciliogenesis. Our results indicated that CEP89 phosphorylation was not correlated with ciliogenesis (Fig. 2G). We also performed a TTBK2 in vitro kinase assay to explore the possible kinase-substrate relationship between TTBK2 and CEP89 by using TTBK2 purified from 293T cells as the enzyme and His-CEP89 purified from bacteria as the substrate. We found TTBK2 only phosphorylated CEP83, not CEP89 in the in vitro kinase assay. Thus, our results indicated that CEP89 was not a TTBK2 direct substrate.



2. TTBK2-dependent CEP164 phosphorylation sites had already been mapped by the paper published in PNAS (Cajanek and Nigg, 2014). Thus, we generated the wild-type CEP164 (CEP164^{WT}) and CEP164 phospho-inactive mutant (CEP164^{6A}) that tagged with Myc epitope at their C-terminus. We also generated *CEP164-/-* RPE1 cells and re-expressed Myc-CEP164^{WT} and Myc-CEP164^{6A} in *CEP164-/-* cells to understand the role of CEP164 phosphorylation in ciliogenesis. When we induced cilia formation by serum starvation for 2 days, we found that Myc-CEP164^{6A} expressing cells still formed cilia. In addition, the ciliated percentage in Myc-CEP164^{WT} or Myc-CEP164^{6A} expressing cells showed no dramatic difference. It is possible that we didn't mutate all the TTBK2-dependent phosphorylation sites on CEP164. It is also possible that TTBK2-dependent CEP164 phosphorylation is not important for ciliogenesis. In order to understand the influence of CEP164 phosphorylation in ciliogenesis, it would be necessary to understand the relationship between CEP164 phosphorylation and ciliogenesis. In addition, generation of CEP164 phospho-specific antibodies would also be required to known whether CEP164 phosphorylation happens during ciliogenesis.



Using super-resolution microscopy, Lo et al analyzed the recruitment and redistribution of TTBK2 from periphery toward the root of mother centriole upon serum starvation. However this was shown in a paper from the same group early this year (Yang et al., 2018, figure 4) and the basic observation was made in Goetz et al., 2012.

Based on the dSTORM images of TTBK2 that we published earlier (Yang et al., 2018), we continued studying the distribution of TTBK2 at centriole distal appendages and identified CEP83 as a TTBK2 substrate (Yang et al., 2018). At that time, we noticed that the TTBK2 signal was different in proliferating and G1-arrested cells. This motivated us to further map the detailed localization of TTBK2 by comparing TTBK2 signals with proteins at the centriole distal-end in proliferating and serum-starved cells (Figs. 1D and 1I). Further analysis of this phenotype in our latest paper (Lo et al), shows that the distribution of CEP164 and CEP83 does not changed upon serum starvation, suggesting that the structure of centriole distal appendages is not altered during ciliogenesis (Figs. 1D and 1I). This would indicate that the redistribution of TTBK2 during ciliogenesis is not due to changes in the structure of distal appendages (Figs. 1D and 1I). In addition, our dSTORM images also showed that the kinase activity of TTBK2 didn't affect its redistribution at the centrioles upon serum starvation (Figs. 1I-1J). Thus, in this paper (Lo et al), we have extended our work based on the redistribution of TTBK2 and characterized its functionality during ciliogenesis and we have uncovered the mechanistic role of CEP83 phosphorylation by TTBK2.

An important, but challenging question is where and when does phosphorylation of CEP83 take place in the cell. The authors only identified the four peptides phosphorylated when TTBK2 is over-expressed but not in the control samples, which indicates the phosphorylation level of CEP83 in vivo is low. What is the phosphorylation state of CEP83 in resting and dividing cells? Ideally, an antibody that recognizes phospho-CEP83 could answer these and related questions.

We thank the reviewer for suggesting to generate the phospho-CEP83 antibodies. In our revision, we successfully generated two anti-phospho-CEP83 antibodies (anti-phospo-CEP83^{Ser29} and anti-phospho-CEP83^{Thr292}) to answer where and when CEP83 phosphorylation took place in cells (please also see our new item, point 2). Our results also indicate that the level of CEP83 phosphorylation in dividing cells is low based on the intensity quantification of phospho-CEP83 signals at the centrioles (Figs. 3G and 3H). Upon inducing ciliogenesis by serum starvation, CEP83 phosphorylation (at CEP83^{Ser29} and CEP83^{Thr292}) increases at the cilia base in a TTBK2-dependent manner (Figs. 3I and 3J).

The phosphorylation-deficient mutant form CEP834A still allows cilia to form at reduced frequency (25% of cells are ciliated). To account for the deficit in cilia formation in the CEP834A mutant, the author example two processes important in cilia initiation: removal of CP110 and association with ciliary vesicles. Both process are reduced, but not abolished, in the mutant. Together with the identification of other possible TTBK2 phosphorylation sites, this suggests some overlap in function between TTBK2 targets. This would be very interesting to explore.

We agreed with the reviewer that some overlap in function between TTBK2 targets might be possible. It has been known that CEP164 mediates vesicular docking to the mother centriole (Schmidt et al., 2012). When we co-expressed TTBK2 in 293T cells, we also observed upward electrophoretic mobility shift of CEP164 (Fig. S1). As I mention

above, we still do not have a clear answer with the influence of TTBK2-dependen CEP164 phosphorylation in ciliogenesis (with the figure provided above). More experiments are required to understand the possible overlap in function between TTBK2 and CEP164.

A recent study identifies MPP9 as a TTBK2 substrate associated (Huang et al., 2018). At the onset of ciliogenesis, TTBK2-dependent MPP9 phosphorylation promotes MPP9 degradation through the ubiquitin-proteasome system. The degradation of MPP9 then facilitates CP110 removal and subsequent cilia initiation. Since CEP83 has been shown to be required for the recruitment of other proteins to the distal ends of centrioles, CEP83 phosphorylation might facilitate the recruitment of MPP9 to centrioles to be phosphorylated by TTBK2 and targeted for degradation. In addition, the TTBK2-dependent CEP83 phosphorylation might also recruit cilia-specific E3 ligases to the mother centrioles promoting the degradation of MPP9 and subsequent CP110 removal.

Minor concerns:

(1) Fig 5B WB result shows the expression of WT CEP83 protein in Cep83 ko cells. However, in the blot of CEP83 panel there appears to be endogenous CEP83 protein band (lower band in the top blot), which should be absent in CEP83 ko cells.

In order to prove that endogenous CEP83 was inactive in those CEP83 re-expressing cells, we genotyped CEP83^{WT}, CEP83^{4A} and CEP83^{DEED} expressing cell lines using primers that only amplified the genome locus of endogenous *CEP83* gene. Both primers were designed to target the intron region of *CEP83* gene. Results from our genotyping experiments indicated that the genome locus of endogenous *CEP83* gene in all these CEP83 re-expressing cell lines was the same as the genome locus of endogenous *CEP83* gene in *CEP83-/-* cells (Fig. S5B). It indicated that endogenous CEP83 was inactive in all those cell lines. The band referred to by the reviewer should be due to a cleavage or degradation product of the Flag-tagged CEP83 expression construct.

(2) In the Fig 2B phos-tag gel blot, co-expressed TTBK2FL induced a phosphorylation-dependent shift in about 50% of CEP83 protein to be shifted (phosphorylated), while in the similar experiment in C, 100% is shifted. What is the basis of this difference?

Since we performed the experiment by transiently expressing TTBK2 and CEP83 in 293T cells, we believed that the percentage difference of the gel shift on CEP83 is due to the different ratio of protein expression between TTBK2 and CEP83. In the western bots of total cell lysates, the level of TTBK2 expression in the figure 2B was relatively lower than in figure 2C. The levels of TTBK2 expression could explain the differences in CEP83 phosphorylation between figures 2B and figure 2C.

(3) Figure 1A, use "pGlu-Tub" instead of "Glu-Tub" to label Polyglutamylation tubulin. Detyrosinated tubulin also designated as Glu-Tub.

Fixed. Thanks.

(4) Fig 2D, F. The molecular weights of CEP83 and 89 are not consistent.

We went back to check our blots and found that we run different percentage of SDS-PAGE gels in the figure 2D (now figure 2E) and figure 2F (now figure 2G) when we performed CEP83 western blot. The figure 2E was run in the 8 % SDS-PAGE and the figure 2F was run in the 10 % SDS-PAGE. Since the molecular weight of CEP83 and CEP89 are around 90 kDa, CEP83 and CEP89 are better separated by using 8% SDS-PAGE.

(5) Figure 5K middle panel, the zoom in cropped box is shifted.

Fixed. Thanks.

(6) There is no antibody information on Myo-Va in the Supplementary Table 2.

Added. Thanks.

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

Major point

(1) In figure 6D, authors created a phosphorylation-defective mutant (4A) and showed that the 4A mutant partially failed to rescue a ciliation defect of CEP83 knockout cells. However, a possible explanation for the failure is that the substitutions from serine/threonine to alanine might partially disrupt the structure of CEP83, and thus results in partial dysfunction of the protein. Also, the phospho-mimic mutant (DEED) in this figure added little to the conclusion.

To strengthen the conclusion, it would be valuable to perform a rescue experiment with the phospho-mimic mutant (DEED) in proliferating cells. It would greatly strengthen the conclusion, if the phospho-mimic mutant is sufficient for removing CP110 and/or recruiting ciliary vesicle, thus for inducing cilium formation.

Thanks for the comments and nice suggestions. We performed the immunostaining of CP110 and Myosin-Va in proliferating cells during our revision (please also see our new item, point 4). In comparison with $CEP83^{WT}$ and $CEP83^{4A}$, $CEP83^{DEED}$ expressing cells showed a higher percentage of ciliated cells (Fig. 6D). In addition, $CEP83^{DEED}$ expressing cells also showed more CP110 removed from mother centrioles and contained more ciliary vesicles when compared to the $CEP83^{WT}$ and $CEP83^{4A}$ expressing cells (Figs. 7F and 7G). These data further strengthened our conclusion that CEP83 phosphorylation promotes CP110 removal and membrane vesicle docking.

(2) The authors conclude that phosphorylation of CEP83 is important for CP110 removal and ciliary vesicle docking. I think the most likely possibility is that the phosphorylated CEP83 regulates those steps via other distal appendage proteins because :

i) Based on classical electron microscopy studies, ciliary vesicle is recruited to the middle-distal region of the distal appendage, whereas CEP83 localizes to the proximal region of the distal appendage.

ii) A distal appendage protein, CEP164, regulates both CP110 removal and ciliary vesicle docking. Further, CP110 removal might be a downstream of ciliary vesicle docking, as shown in the previous paper (https://www.ncbi.nlm.nih.gov/pubmed/25686250).

iii) CEP83 is required for the localization of other distal appendage proteins.

In that sense, it would be valuable to test whether the localization of other distal appendage proteins is affected in the CEP83 knockout cells expressing phosphorylation defective (4A)-CEP83. The authors might have addressed this in the Fig. 6B, but some quantification is needed. All other distal appendage proteins, including LRRC45, should be tested. Moreover, authors should test the localization of IFT-B and transition zone components, as their localization is dependent on CEP164 http://jcb.rupress.org/content/199/7/1083.

In our revision, we analyzed key steps during ciliogenesis in CEP83^{WT}, CEP83^{4A}, and CEP83^{DEED} expressing cells. Immunostaining was performed in proliferating and serum-deprived cells to understand how CEP83 phosphorylation affected CP110 removal and membrane vesicle docking.

We first **quantified the fluorescent intensities** of **all proteins at distal appendages** (Figs. 6E&6F and Figs. 5D). The intensities of proteins at centriole distal appendages including LRRC45 showed no difference in CEP83^{WT}, CEP83^{4A}, and CEP83^{DEED} expressing cells, indicating the integrity of centriole distal appendages was not affected by CEP83 phosphorylation (Figs. 6E&6F and Figs. 5D).

We also quantified **proteins recruited to centriole distal appendages by CEP164** (Figs. 6E&6F and Figs. 5D). TTBK2 and ITF88 **(IFT-B complex)** are known to be recruited to centriole distal appendages via CEP164. Since CEP83 phosphorylation does not affect the recruitment of CEP164 to distal appendages, our results also show that the recruitment of IFT88 and TTBK2 are not affected in CEP83^{WT}, CEP83^{4A}, and CEP83^{DEED} expressing cells (Figs. 7A, 7B, and S5D).

Both CP110 removal and the docking of membrane vesicles have been shown to promote transition zone assembly. According to our results, both of these processes are affected by TTBK2-dependent CEP83 phosphorylation. Thus, we examined **the assembly of the transition zone** by testing the localization of several transition zone components. Our results showed that the establishment of the transition zone was affected by CEP83 phosphorylation (Fig. 7E).

Thus, our quantifications with proteins at centriole distal appendages show that CEP83 doesn't affect the localizations of other distal appendage proteins. In addition our results also indicate that localization of IFT-B and transition zone components recruited by CEP164 is not affected by CEP83 phosphorylation.

A significant question is how phosphorylated CEP83 can promote CP110 removal. In the discussion, we hypothesized that TTBK2-dependent CEP83 phosphorylation might regulate ciliogenesis by affecting the removal of MPP9 at the centriole distal-end. Both CEP83 and MPP9 are TTBK2 substrates. It has been shown that TTBK2-dependent MPP9 phosphorylation promotes MPP9 degradation at the onset of ciliogenesis. The

degradation of MPP9 then facilitates CP110 removal and subsequent cilia initiation (Huang et al., 2018). Since CEP83 has been shown to be required for the recruitment of other proteins to the distal ends of centrioles, CEP83 phosphorylation might facilitate the recruitment of MPP9 to centrioles to be phosphorylated by TTBK2 and targeted for degradation.

Minor point

(1) Page 3, line 6. Although authors mentioned LRRC45 in the discussion, LRRC45 should be mentioned in the introduction as well. Is TTBK2 not a distal appendage protein?

We've included the LRRC45 in the introduction. We also apologize for not including TTBK2 in list of centriole distal appendage proteins, and have now added it.

(2) Page 3, line 7. In this context, authors should refer the papers that discovered the distal appendage proteins (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2064767/ and https://www.ncbi.nlm.nih.gov/pubmed/21976302) rather than the papers that have listed distal appendage proteins on them, specifically (Lu et al., 2015) and (Schmidt et al., 2012).

We apologize for missing these key references, and have now included them.

(3) page 3, line 3. MMP9 paper should be mentioned not only in the discussion but also in the introduction.

We have included it in the introduction. Thanks.

(4) page 5 lines 1-3 and page 11 lines 2-4. The authors should discuss a potential mechanism for the TTBK2 redistribution.
Given that CEP164 is absolutely required for TTBK2 localization, CEP164 distribution might also change upon serum deprivation. The distribution pattern shown looks highly similar to that of CEP164 (Fig. S2 of Yang et al., 2018). It is not clear if this CEP164 image was taken under serum-starved condition, but if this is the case, it would be very interesting to see the localization of CEP164 in proliferating cells.
Another possibility is that two peaks (150 nm and 225 nm) of CEP83 radial diameter cannot be observed in proliferating cells, because of the weaker signal (Fig. 1C, right graph and Fig. 1E).

We performed several experiments to test the potential mechanism for TTBK2 redistribution upon serum starvation. After generating *TTBK2-/-* cells, the requirement of TTBK2 kinase activity in cilia formation was confirmed by re-expressing of full-length TTBK2 ($TTBK2^{FL}$) or TTBK2 catalytically inactive mutant ($TTBK2^{KD}$) in *TTBK2-/-* cells. The dSTORM images showed that both $TTBK2^{FL}$ and $TTBK2^{KD}$ formed smaller rings around the mother centrioles upon serum starvation, revealing that TTBK2 kinase activity didn't affect TTBK2 redistribution at the centriole distal appendages during ciliogenesis (Figs. 1H and 1I).

Since the CEP164 distribution pattern was taken under serum-starved condition (Fig.

S2 of Yang et al., 2018) (Yang et al., 2018), we have now also provided the dSTORM images of CEP164 and CEP83 in proliferating and G1-arrested conditions (Figs. 1D and 1I). The results showed no difference on CEP164 and CEP83 patterns at the centrioles in proliferating and serum-starved cells. The dSTORM images showed that the distributions of CEP164 and CEP83 at the DAs were not altered upon serum withdrawal, indicating the DAs structure was not altered upon serum starvation (Figs. 1D and 1I). Since distal appendage proteins did not seem to redistribute upon serum starvation, it is still unclear how TTBK2 relocalization is regulated

We rigorously examined whether we the CEP83 signal could be present at the radial location of 225 nm. However, we did not find any CEP83 signal around this area (Fig. 1I).

(5) page 12 line 24 and page 13 line 1. "nephronophthisis (NHPH18)" should be "nephronophthisis (NPHP18)"

Fixed. Thanks.

(6) page 13. Authors should greatly improve their methods section to ensure reproducibility. Much important information are missing.

New details have been added to “Materials and methods”. Thanks.

(A) Methods for "purification of recombinant protein", "co-immunoprecipitation", "quantification of signal intensity" are missing.

We have included it in the material and method section. Thanks.

Purification of recombinant proteins

For recombinant protein purification, constructs were transformed into *Escherichia coli* Rosetta2 (DE3) strain (Novagen). Cells were cultured in LB media at 37 °C until O.D.600 reached 0.5–0.6. Expression of the recombinant proteins were then induced at 25°C for 16 h by adding isopropyl β-D-thiogalactopyranoside (IPTG) (0.2mM for GST fusion proteins and 2mM for His fusion proteins). Purification of the His and GST fusion proteins were performed as described (Tsai et al., 2000).

Immunoprecipitation

A 100-mm dish of transfected cells were lysed in the buffer that contain 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1%, Nonidet P-40, and 0.5% sodium deoxycholate together with phosphatase and protease inhibitor. 1mg of cell lysates were suspended in 1 ml lysis buffer and incubated with 5 µl of anti-FLAG M2 beads (Sigma-Aldrich) for 2 h at 4°C under gentle rotation. Beads were then washed three times with the lysis buffer that contained protease and phosphatase inhibitors. The immunocomplex were eluted by SDS sample buffer and separated by gels for western blot analysis.

Quantification of images from immunostaining and immunoblots

For quantification of fluorescent intensity with proteins at the centrioles, all cells were treated the same during the process of immunostaining and image acquisition. The ZEN

software (Carl Zeiss) was used to analyze the image intensity. The same setting was applied to all images. A circle was drawn surrounding the centrioles and the total pixel value of the marked region was then measured. The signal ratio of the marked region over the proteins at the centrioles was then normalized to the control group. ImageJ software (National Institutes of Health) was used to quantify the bands of immunoblots. A rectangle was drawn surrounding the target band. Results were expressed as density means ± SD by normalizing to the control group. All the quantifications were obtained from at least three independent experiments.

(B) page 13 line 13. "complete medium". Which media? Did authors use the same media for all cell lines?

Detail information regarding the medium that we used in cell culture has been added in the materials and methods. Thanks.

Cell culture and reagent

293T, 293FT, and U2OS were cultured in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The human telomerase-immortalized retinal pigment epithelial cells (hTERT-RPE1 or RPE1) were cultured in DME/F-12 (1:1) medium supplemented with 10% FBS and 1% penicillin-streptomycin.

(C) page 13 line 14. "transient transfections" What is the method for a transient transfection? How many cells were plated into which dish? How much DNA and transfection reagent were used?

We used liposome method for the transfection. The detail method regarding the cell numbers, and DNA amount are showed in the methods in the revised manuscript.

Transient transfection

Transient transfections were performed using T-Pro NTR II transfection reagents (T-Pro Biotechnology). $3X10^6$ of 293T or U2OS cells were plated on 100-mm plate for overnight. Cells were transfected with 10 μg of expression constructs according to the manufacturer's instructions. For transient transfection, cells were harvested 48 h after transfection.

(D) How did authors create a retrovirus? Which cells were used? How much of puromycin was used to select the cell lines?

We apologize for not including the method for generating retrovirus in the submitted manuscript, the revised manuscript has included a new section regarding how we generated retrovirus and selected cells by puromycin.

Retrovirus production and infection

$5x10^5$ of 293FT cells were plated on 60-mm dish using T-Pro NTR II transfection reagents with following plasmids: 1.5 μg of V-SVG, 2.5 μg pCMV-gag-pol and 4μg of the pBabe-puro3 based constructs. The supernatant containing viral particles was harvested 48 h

after transfection. Virus containing media was centrifuged at 1000rpm for 5 minutes and passed through a 0.45µm filter (Sarstedt). For the infection of RPE1 cells, 5×10^5 of RPE1 cells were seeded onto a 60-mm plate the night prior to infection and incubated with 3 ml of viral stock. The medium was changed to fresh culture medium 18 to 24 h after infection. Two days after infection, cells were selected and maintained in culture medium containing 2 µg/ml of puromycin (Sigma-Aldrich).

(E) page 13 lines 21, 23 and 27. "pcDNA3-FH or pRK5M vector", "pBabe-puro3", "pET32a". Are there references and/or catalog numbers for these vectors? Does pcDNA3-FH vector contain FLAG-HA tag at N-terminus or C-terminus? What was the pRK5M vector used for? (i.e. In which experiments did authors use this vector?).

We've now made it clearly in our materials and methods. The pRK5M vector contained sequence for expression of Myc-epitope at protein C-terminus (Feng et al., 1995). The $pcDNA_3$-FH vector was derived from $pcDNA_3$ (Thermo Fisher Scientific) but contained sequences for Flag and HA epitope-tag between the HindIII and BamHI cloning sites. Thus, the expressing proteins expressed Flag and HA epitope tag at the N-terminus. The FH-tagged TTBK2 and CEP83 fragments were also sub-cloned into pBabe-puro3 vector (Morgenstern and Land, 1990) so that proteins could be stably expressed in RPE1 cells.

We cloned CEP83 and TTBK2 fragments in pRK5M vector for performing the co-IP experiments to check TTBK2-CEP83 interaction (CEP83 was tagged with Myc and TTBK 2 was tagged with FH; Figs. 4A, 4C, and Fig. S3) and for analyzing CEP83 phosphorylation (TTBK2 was tagged with Myc and CEP83 was tagged with FH; Figs. 3A and 3D).

(F) page 14 line 19. "incubating with the indicated primary antibodies". How long?

The primary antibodies were all diluted in blocking buffer (3% bovine serum albumin (w/v) and 0.1% Triton X-100 in PBS) and incubated for 2 h at RT. This is now clearly stated in the material and methods section.

(G) page 14 line 19 "Secondary antibodies were all from molecular probes". Molecular probes provide a variety of secondary antibodies. Authors should specify fluorophores and the catalog number of each secondary antibody.

Alexa Fluor 488–, 594–, or 680-conjugated goat secondary antibodies were used at 1:500 dilution (Molecular probes) and incubated for 1 h at RT. Added. Thanks.

(H) page 14 line 23. Objective lenses (with NA) are missing.

We used the Plan-NEOFLUAR ×100 (1.3 NA) oil-immersion objective. Added. Thanks.

(I) page 15 line 2. Catalog numbers for PVDF membrane are missing. Depending on the detection method (chemiluminescence or fluorescence), a specific PVDF membrane has to be chosen.

The PVDF membrane we used was Amersham™ Hybond™ P 0.45µm from GE

Healthcare Life Science. We have included it in the material and method section.

(J) page 15 line 4. Catalog numbers for alkaline phosphatase is missing. NEB provides several alkaline phosphatases. Which one?

The alkaline phosphatase was the calf intestinal alkaline phosphatase (CIP) from New England BioLabs. We have included it in the material and method section. Thanks.

(K) page 15 "dSTORM" Which chamber was used? How many cells were plated? How long the samples were incubated with primary/secondary antibodies? What was the concentration of primary/secondary antibodies? This information is very important because inefficient staining could result in the defect of a super-resolved structure (https://www.ncbi.nlm.nih.gov/pubmed/22735543)
Which fluorophores were used? Alexa647 for single color? And Alexa647 and Cy3B for 2 color?
Which laser lines? Was the activation laser used? How many frames were typically recorded? What is the pixel size of the final image?
How did author calculate the radial diameters?

Thanks for the comments. All of the immunostaining in the paper used the same protocol. Cells were grown on 0.1 mg/ml of poly-L-lysine coated coverslips and fixed with methanol at −20□C for 15 min. The cells were incubated in the blocking buffer that contained 3% bovine serum albumin (w/v) and 0.1% Triton X-100 in PBS for 30 min. Primary antibodies were all diluted in blocking buffer and incubated for 2 h at room temperature. Dilution for each antibody was shown in the Supplemental Experimental Procedures. Secondary antibodies were used at 1:500 dilution and incubated for 1 h at room temperature. The dSTORM Superresolution images were performed on a modified setup based on a commercial inverted microscope (Eclipse Ti-E, Nikon), with 100x 1.49 NA oil-immersion objective (Yang et al., 2018). For single color imaging, a 637 nm laser (OBIS 637 LX 140 mW, Coherent) was utilized for the excitation of Alexa Flour 647. For dual color imaging, Alexa 647 imaging was first performed and then the second channel imaging of Cy3B was sequentially acquired with a 561 nm light (Jive 561 150 mW, Cobolt). The excitation lights were operated at 3-5 KWcm-2. A weak 405 nm light (OBIS 405 LX 100 mW, Coherent) was introduced to convert fluorophores from a dark state to fluorescence state. The fluorescence emission was spectrally cleaned with a bandpass filters (700/75 and 593/40 for Alexa 647 and Cy3B, respectively; Chroma) prior to readout by an electron-multiplying charge-coupled device (EMCCD) camera (Evolve 512 Delta, Photometrics). Fiducial markers (Tetraspeck, 0.1µm, T7279) were used to record the drift during the imaging for the post-acquisition linearity correction. Typically, 10000-20000 frames were taken at 50 frames per second. The position of each single-molecule event was analyzed using Metamorph Superresolution Module (Molecular Devices) and the final rendered pixel size of dSTORM images was 11.625 nm. For dSTORM, samples was mounted with an imaging chamber (CM-B18-1; Live Cell Instrument) and incubated in the imaging buffer containing 50mM Tris-HCl, 10mM NaCl (TN) buffer at pH 8.0, and an oxygen-scavenging system consisting of 100 mM mercaptoethylamine at pH 8.0, 0.5 mg mL−1 glucose oxidase, 40 µg mL−1 catalase, and

10% glucose (Sigma-Aldrich). To determine the radial distribution of CEP83/CEP164/TTBK2 in Figure 2, a centre of individual puncta of protein distribution was first fitted and then their radial positions were described with a distance with respect to the centre. All this information was included in detail in the revised material and methods section.

(L) page 15 line 23 "Tetraspeck" What diameter? Please specify the catalog number.

The diameter of Tetraspeck is 0.1µm and the catalog number is T7279 from Thermo Fisher. We have included it in the material and method section.

(M) page 15 line 25. What are the concentrations of Tris-HCl and NaCl.

The concentration of Tris-HCL and NaCl was 50 mM and 10 mM, respectively.

(N) page 16 line 2. How many 293 cells were plated in which dish? What volume of M2 beads were used? Was the kinase assay performed on the beads? Or FLAG-tagged TTBK2 was eluted with FLAG-peptide? Were the beads washed with kinase buffer before adding CEP83? How much purified CEP83 was added?

(O) page 16 line 9. Authors should also include how the phosphorylation was detected. (Added sample buffer, performed SDS-PAGE etc...).

A clear description on how we transfected FH-TTBK2 in 293T cells, purified FH-TTBK2 by M2 beads, and carried out the TTBK2 in vitro kinase assay have been included in the material and methods sections.

The immunoprecipitation of FH-TTBK2 fro TTBK2 kinase assay was performed as following:

Various FH-TTBK2 proteins were transiently expressed in 293T cells for 48 h. A 100-mm dish of FH-TTBK2 transfected cells were lysed in the buffer that contain 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1%, Nonidet P-40, and 0.5% sodium deoxycholate together with phosphatase and protease inhibitor. 1mg of cell lysates were suspended in 1 ml lysis buffer and incubated with 5 µl of anti-FLAG M2 beads (Sigma-Aldrich) for 2 h at 4°C under gentle rotation.

The TTBK2 in vitro kinase assay was performed as following:

Beads were washed three times by TBS buffer (50mM Tris-HCl and 150mM NaCl at PH7.4) followed by TTBK2 kinase reaction buffer (50 mM Tris pH7.4 and 10 mM $MgCl_2$) before performing TTBK2 kinase assay. TTBK2 kinase assay was performed by incubating TTBK2 protein on the beads with 5µg of recombinant His-CEP83^{WT} or His-CEP83^{4A} for 30 min at RT in 30 µl of kinase reaction buffer that contained 100 ng ATP-gamma-S (ab138911; Abcam). After incubation, 1µl of the 50 mM p-nitrobenzyl mesylate (ab138910; Abcam) was added to the kinase reaction for 90 minutes at RT to stop kinase reaction. Proteins were separated by SDS-PAGE and then transferred to the NC membranes (GE healthcare) for western blot. The immunoreactive bands were detected by Odyssey® Image Systems (LI-COR). The gel was also stained with Coomassie blue (CBB) to ascertain migration of the proteins. For TTBK2 in vitro kinase assay that used

GST-TTBK2 (a.a. 1-622)^{WT} and GST-TTBK2(a.a. 1-622)^{KD} purified from bacteria, 0.5μg of GST-TTBK2 (a.a. 1-622)^{WT} or GST-TTBK2(a.a. 1-622)^{KD} and 5μg of CEP83 were added in the 30 μl of kinase reaction buffer for 30 min at room temperature that contained 100 ng ATP-gamma-S.

(7) page 25 line 9. "FLAG-tagged CEP83" This seems like HA-tagged CEP83, otherwise subsequent detection with HA (Fig. 4D) does not make sense.

We apologize for this confusion. We cloned CEP83 cDNA in the pcDNA3-FH vector. The pcDNA$_3$-FH expression vector was derived from pcDNA$_3$ (Invitrogen) but contained the sequences for a Flag and HA epitope tag between the HindIII and BamHI sites. Thus, the epitope tagged proteins could be detected by both Flag or HA antibodies. We've written it in detail in the materials. We also made our labels clear in the results figures and figure legends.

(8) page 26 line 2. "Cells were serum starved to induce cilia formation" How long?

RPE1 cells were serum starved for 2 days to induced cilia formation. We have now added this in the figure legend.

(9) page 26 line 14. "G1-arrested cells" How long were the cells serum-starved?

To generate G1-arrested cells, RPE1 cells were serum starved for 2 days. We have now added this information in the figure legends.

(10) Figure 2A and Figure 5B. Authors detected FBF1 at 75 kDa (Fig.2A) and 55 kDa (Fig. 5B). Please confirm that authors actually detected FBF1, because the molecular weight of the main isoform of this protein is 125 kDa. The previous paper showed FBF1 protein around 130 kDa. https://www.nature.com/articles/ncomms3750

We apologize for this mistake. In deed, the actual size of the main FBF1 isoform is around 140kDa. That has also been confirmed in the paper published in Nature communication (Figure 5H)(Wei et al., 2013). We have confirmed the detection of endogenous FBF1 shown in figure 2A and figure 5B. Thanks.

(11) Supplementary table 2. "anti-centrin 2" Authors seemed to use centrin antibody, but not centrin 2 antibody. The catalog number indicates centrin antibody.

Fixed. Thanks.

(12) The anti-Myosin Va antibody is missing.

Added. Thanks.

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

In the manuscript entitled "TTBK2 phosphorylates CEP83 in promoting cilia initiation", the authors present compelling evidence that the kinase activity of TTBK2 promote ciliogenesis in phosphorylating CEP83 at centriole distal appendages. The study is comprehensive, starting with the observation that TTBK2 redistributes its localization at centriole distal appendages closes to CEP83 during ciliogenesis. The authors then identify

CEP83 as a novel substrate for TTBK2 and characterize four phosphorylation sites. They show that TTBK2-dependent CEP83 phosphorylation is required for the initial stages of ciliogenesis in RPE1 cells, in particular the formation of the ciliary vesicle and the removal of CP110 from the centriole distal end. The authors also mapped the domain in CEP83 required for targeting of the distal appendage components and recruitment of TTBK2 to the mother centriole.

Overall the manuscript is well written and quite informative. This is a significant finding regarding cilia initiation mechanism involving TTBK2 via its kinase activity in phosphorylating DAPs at the centriole. The methods used (STORM, SIM, Mass spectrometry and CRISPR-Cas RPE1 cell lines) are state of the art and the results support the conclusions made.

However, my main concern is the absence of quantification of Western blot analyses (with statistical analysis) and of fluorescence intensity of the proteins recruited at the centrioles presented throughout the manuscript (Fig. 2A, F, 3A, D-G, 6C and Fig. 5C-J, 6B, C respectively).

In the revised manuscript, we quantified bands from our western blots as well as the fluorescent intensity with the proteins at the centrioles (Figs. 2A, 2G, 3A-3F, 3H, 3J, 4A, 4J, 5D, 5G-5L 6B, 6F, 7B, 7E, S2E, S3A, S3D, S4A, and S4B).

Specific points:

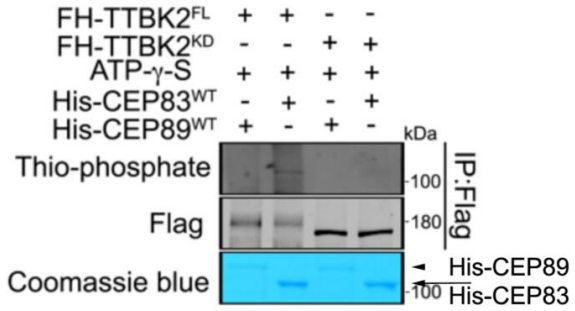
1- Fig.2A: Quantification of WB analysis of centriolar proteins is required, in particular for the bands corresponding to CEP164 and FBF1 that seem slightly increased in serum starved conditions.

Thanks for the comment and suggested experiments. After quantifying the image intensities of the western blots from at least three independent experiments, the results showed that the expression of these centriolar proteins was not affected by serum starvation (Fig. 2A).

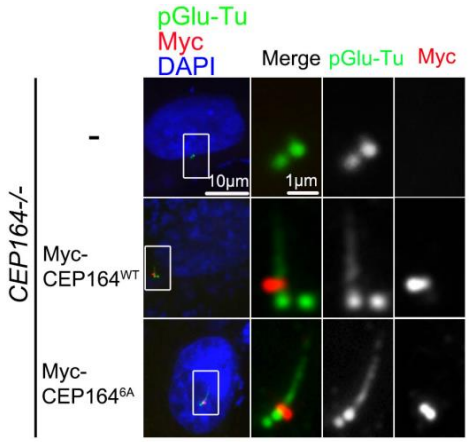
2- Fig. 2B-Fig. S1: The authors observe that " CEP83 showed an upward gel mobility shift on reducing gel and phospho-tag gel when full-length TTBK2". Is the upward gel mobility shift specific to CEP83? Have other DAP components been tested? Why biochemistry has been performed on overexpressed proteins only? What about the endogenous CEP83?

We performed the gel mobility shift analysis of endogenous CEP83 (Fig. 2D).

In addition to CEP83, we also observed an upward gel mobility shift of CEP89 and CEP164 on the reducing gels (Fig. S1). However, the reason that we didn't continue analyzing the effect of TTBK2 on CEP89 or CEP164 phosphorylation was due to the following reasons: First, we analyzed CEP89 phosphorylation during ciliogenesis and our results indicated that CEP89 phosphorylation did not correlated with ciliogenesis (Fig. 2G). Second, in our in vitro kinase assays using TTBK2 purified from 293T cells (as the enzyme) and His-CEP89 purified from bacteria (as the substrate) we found no direct phosphorylation of CEP89 by TTBK2.



We tried to understand the effect of the TTBK2-dependent CEP164 phosphorylation in ciliogenesis. The sites on CEP164 that are phosphorylated by TTBK2 were previously mapped (Cajanek and Nigg, 2014). Thus, we generated wild-type CEP164 (CEP164^{WT}) and a CEP164 phospho-inactive mutant (CEP164^{6A}) that tagged with the Myc epitope at their C-terminus. We then generated *CEP164-/-* RPE1 cells and re-expressed Myc-CEP164^{WT} and Myc-CEP164^{6A} in *CEP64-/-* cells to examine the role of CEP164 phosphorylation in ciliogenesis. When we induced cilia formation by serum starvation for 2 days, we found that Myc-CEP164^{6A} expressing cells still formed cilia (picture below). In addition, the ciliated percentage in Myc-CEP164^{WT} or Myc-CEP164^{6A} expressing cells showed no dramatically difference. It could be possible that we didn't mutate all the TTBK2-dependent phosphorylation sites on CEP164. In order to understand the role of CEP164 phosphorylation in ciliogenesis, it would be necessary to identify all the phosphorylation sites on CEP164 that was mediated by TTBK2. In addition, generation of CEP164 phospho-specific antibodies would also be important for us to known whether CEP164 phosphorylation happens and is important for ciliogenesis.



3- Fig. 2D: The meaning "FH" as Flag tagged should be indicated in the Figure legend or in the text. Why the authors used so many different constructs in not clear.

We apologize for the unclear labels. Most of CEP83 and TTBK2 cDNA were cloned into pcDNA$_3$-FH vector. The pcDNA$_3$-FH vector was derived from pcDNA$_3$ but contained sequences for Flag and HA epitope tag between the HindIII and BamHI cloning sites. Thus, the tagged proteins could be detected by a Flag or HA antibody. We've now

written this clearly in our results, material and methods section, and in the figure legends.

4- Figure S2A. The authors claim that the interaction between CEP83 and TTBK2 "was not relied on the TTBK2 kinase activity, as expression of TTBK2FL and TTBK2KD showed no difference for their interaction." Quantification of several experiments is required as CEP83Myc seems to bind less TTBK2KD than TTBK2FL and especially as the amount of TTBK2FL and TTBK2KD precipitated is not equivalent.

We have quantified the western blots regarding the interaction between CEP83 and $TTBK2^{FL}$ or $TTBK2^{KD}$ from at least three independent experiments. The quantification showed no difference between the CEP83-$TTBK2^{FL}$ and the CEP83-$TTBK2^{KD}$ interaction (Fig. S3A).

5- The authors do not provide evidence of interaction/phosphorylation with the endogenous proteins CEP83 and TTBK2. Why not observe the phosphorylation status of the endogenous CEP83? What about the endogenous interaction? Overexpressed cytosolic proteins in cells expressing the endogenous protein might not be engaged in the interaction. What about the localization and co-localization of TTBK2KD and CEP83 in those cells?

Apologies for this. After trying all the CEP83 and TTBK2 antibodies in our hand, we were unable to immunoprecipitate the endogenous proteins. Thus, our results reflect the work carried out with over-expressed proteins.

To further our understanding of a functional interaction between CEP83 and TTBK2 during ciliogenesis, we generated two phospho-CEP83 antibodies (anti-phospho-$CEP83^{S29}$ and anti-phospho-$CEP83^{T292}$). Please also see the point 2 in our new items. With these new reagents, we showed that the phosphorylated level of endogenous CEP83 at the centrioles was much higher in ciliated cells than in non-ciliated cells (Figs. 3G and 3H). In addition, using these antibodies and TTBK2 knockout cells, our results showed that CEP83 phosphorylation was increased when RPE1 cells were serum deprived to promote ciliogenesis and this was not observed in *TTBK2* knockout cells (Figs. 3F, 3I, and 3J). Collectively, our results indicate that the serum starved-induced endogenous CEP83 phosphorylation is mediated by TTBK2.

To examine the influence of TTBK2 activity in the regulation of its co-localization with CEP83, we re-expressed $TTBK2^{FL}$ and $TTBK2^{KD}$ in *TTBK2* knockout cells (Figs. 1G and 1H). The dSTORM images showed that both $TTBK2^{FL}$ and $TTBK2^{KD}$ formed smaller rings around the mother centrioles upon serum starvation, revealing that TTBK2 kinase activity didn't affect TTBK2 redistribution at the DAs during ciliogenesis (Figs. 1, I and J).

6-The Fig.3D-G lacks quantification. Fig. 3G: the annotation of the "Flag-TTBK2" is missing on the figure legend. Moreover, image corresponding to the Commassie blue coloration lacks annotations: molecular weight marker is missing, CEP83-HIS... What is the size of the HIS-CEP83 products? According to the Fig. 3G corresponding band is

above 100 kDa. Why is it so different from the size of Flag-CEP83 that migrates above 75 kDa in the other Figures?

We have included the quantification for Figures 3D-G (now the Figs. 3A-3E and Fig. S3D).

We apologize for this unclear labeling. Annotations and molecular weight markers were added in the figure legend (Fig. 3E). The CEP83 was constructed in the PET32a vector so that the expressed and purified His-CEP83 contained His-tag at its N-terminus. Since we didn't remove the His-tag from His-CEP83 after purification, the molecular weight of recombinant His-CEP83 protein was about 100kDa (Fig. 3E).

7- Fig.4C: The authors generate RPE1 cell line (wt or SCKT1 KO) stably expressing CEP83-Flag. Same remark as before, why not look at the endogenous CEP83 protein in those cells?

We looked at the endogenous CEP83 phosphorylation in wild-type and *SCLT1-/-* cells by using two phospho-CEP83 antibodies (Figs. 4I and 4J). In *SCLT1* knockout cells, CEP83 phosphorylation could not be detected upon serum starvation (Fig 4I and 4J). Our results supported the conclusion that the recruitment of TTBK2 to distal appendage by CEP164 was required for TTBK2 to phosphorylate CEP83.

8-Fig.S4A: Quantification should be provide.

Provided. Thanks.

9- Fig.5B "Flag and HA-tagged CEP83 was stably expressed in CEP83-/- cells. Western blot analysis was performed with antibodies as indicated." An additional band is detected by the CEP83 antibodies in CEP83-/- cells stably reexpressing CEP83-Flag, which that is not recognized by the Flag antibodies and that may correspond to the endogenous CEP83 protein. It may also be a cleavage/degradation product of the reexpressing CEP83 protein lacking the Flag tag. Same remark for the WB presented in Fig. S6. What about the mRNA expression in CEP83-/- cells? If the Cep83 transcript expression is down in those cells it can be an easier way to confirm the absence of endogenous CEP83.

In order to prove that endogenous CEP83 was inactive in those CEP83 re-expressing cells, we genotyped CEP83^{WT}, CEP83^{4A} and CEP83^{DEED} expressing cell lines using primers that only amplified the genome locus of endogenous *CEP83* gene (both primers were designed to target the intron region of *CEP83* gene). Our results indicate that the genome locus of endogenous *CEP83* gene in all these CEP83 re-expressing cell lines was the same as the genome locus of endogenous *CEP83* gene in *CEP83-/-* cells (Fig. S5B). It indicated that endogenous CEP83 was inactive in all those cell lines. We agree with the reviewer that the band below the Flag-tagged product might be cleaved protein lacking the flag tag.

10- Fig. 5F: The authors use U2OS cells to map the distal appendage-targeting domain in CEP83? Why the use of this additional cell line? This can be confusing to multiply the

number of cell lines.

We generated cell lines that stably expressed CEP83^{WT}, CEP83$^{1-150}$, CEP83$^{1-336}$, CEP83^{151E}, and CEP83^{367E} in *CEP83-/-* RPE1 cells. We also used those cells to show that the distal appendage-targeting domain of CEP83 was at its C-terminus (Figs. 5E-5G). The conclusion was the same as the figures showing in U2OS (Figs. S4E and S4F).

11- Figures 5H and 5I: WB of stable RPE1 cells reexpressing the CEP83 mutants (151E, 367E) should be provide.

Provided (Fig. 5F). Thanks.

12- A quantification of the recruitment of CEP164 and CEP89 (Figures 5H and 5I) and CEP83, CEP164, TTBK2 (Fig. 6B) at the DAP should be provide. Of note, in Fig. 5I, CEP164 inset is different from the CEP164 staining in the merged picture. Most probably a duplication of the HA staining.

We apologized that we duplicated the HA staining. We have now corrected it (Fig. 5I). In addition, quantifications with the images were provided in the revised manuscript (Figs. 5H, 5I, 5J, 6F, and 7B).

As a decrease of CEP164 or CEP89 may altered the integrity of the 9 doublets of centriolar distal appendages, STORM analyses could be helpful to confirm their integrity.

We thank the reviewer for this insightful suggestion. After quantifying the intensity of CEP89, CEP164, and TTBK2 signals at the centrioles, we found that the intensity of CEP164 at the centrioles decreased in CEP83^{151E} expressing cells by comparing with the intensity of CEP164 at the centrioles in CEP83^{WT} expressing cells (Fig. 5I). This indicated the assembly or integrity of centriole distal appendages was affected in the CEP83^{151E} expressing cells. The impaired recruitment of CEP164 to centrioles also reduced the association of TTBK2 with centriole distal appendages and ciliogenesis (Figs. 5J-5L). A description of these results, including the quantification data, has now been included in the paper (Fig 5H-5L).

13- Fig. 6C: The quality of the blot is poor. Quantification of independent experiment should be provided.

We have now replaced this with a good quality western blot (Fig. 6B). In addition, we have also quantified the levels of CEP83 phosphorylation from at least three independent experiments, shown in Figure 6B.

14- In the discussion part, the authors do not discuss how they reconcile the recruitment of TTBK2 by CEP164 at the DAP and the TTBK2 redistribution from the periphery toward the root of centriole distal appendage close to CEP83 to allow it's phosphorylation"?

We have now included this in the discussion. It has been shown that TTBK2 is recruited to the centriole distal appendages by CEP164 (Cajanek and Nigg, 2014; Oda et al., 2014). Our results show that the recruitment of TTBK2 to the centrioles by CEP164 is a prerequisite step for the following TTBK2 relocation toward the root of centriole distal appendages upon ciliogenesis to phosphorylate CEP83 (Fig. 4). We also performed the

sequence analysis of TTBK2 and found that TTBK2 sequence was highly enriched with the serine residues at its C-terminus, which was the region that TTBK2 associated with CEP164. Recently studies have shown that the phosphorylation of serine residues increases the intrinsic propensity of local backbone structure to form the polyproline II helix (He et al., 2016). Thus, it is possible that serum starvation induces the local conformation change of TTBK2 by changing the phosphorylation levels of the serine residues at TTBK2 C-terminus. The change of TTBK2 local conformation dissociates TTBK2 from CEP164 toward CEP83.

Minor changes:

1- Fig4D: "CEP83-Flag" instead of "CEP83-HA".

The CEP83 construct contains both Flag and HA-tag at its N-terminus. We've made our label clearly in the figures (Figs. 4F and 4G). Thanks.

2- Fig.5A: Page 8 line 1, "... a clone of T deletion in both alleles,..." should be rephrasing as "...a clone with a one base pair (T) deletion on both alleles"

Fixed. Thanks.

3- Page 11 line 29; MPP9 is misspelled

Fixed. Thanks.

4- Page 12 and 13: NPHP18 is misspelled

Fixed. Thanks.

References:

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July 1, 2019

Re: JCB manuscript #201811142R

Prof. Won-Jing Wang
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Taiwan

Dear Prof. Wang,

Thank you for submitting your revised manuscript entitled "TTBK2 phosphorylates CEP83 in promoting cilia initiation". The manuscript has been seen by two of the original reviewers whose full comments are appended below. Please note that the original reviewer #2 was not available to re-review the paper. In any case, 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 #1 is largely satisfied with the revision - his/her remaining issues can likely be addressed by additions to the text. Similarly, reviewer #3 has also raised a few points that will require further explanation and/or changes to the text.
With regard to reviewer #2's points, we have gone through your responses and we have the following comments: As indicated in point#2ii by reviewer #2 (in the first round of review), CP110 removal is likely downstream of ciliary vesicle recruitment (PMID: 25686250). You should focus your discussion and Figure 7F and this linear relationship rather than discuss the less persuasive MMP9 story published last year. In addition, you should use the term G0 rather than G1 when talking about serum-starved cells.

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 I will 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
JCB

Tim Spencer, PhD
Deputy Editor
Journal of Cell Biology

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

The authors have been very responsive to the comments of the reviewers. In particular, they carried out substantive new experiments that address important points.

They now show:
1) Using proteins purified from E. coli, they document the direct phosphorylation o CEP83 by TTBKs
2) They generated TTBK2-/- RPE1 cells and were able to show the CEP83 phosphorylation depends on TTBK2.
3) They made two phospho-specific antibodies against two of the four CEP83 sites (S29 and T292) and used vitro kinase assay and IF experiments to support the hypothesis that phosphorylation of each site is mediated by TTBK2.

The experiments provide convincing evidence that CEP83 is a bonafide TTBK2 target.

However, a weakness of the revised paper is that they do not address how significant their findings are for the regulation of cilia formation in vivo. For example, the authors do not carry out experiments that would compare the functions of two TTBK2 targets, CEP83 and CEP164. In their final model (Figure 7H) they infer that phosphorylation of CEP83 by TTBK2 has two roles: removal of CP110 and ciliary vesicle docking. The data supporting this model are presented in two bar graphs (Fig 7F, G), with marginal significance (*). But the raw data showing an effect on ciliary vesicle association are limited to staining with Myosin Va (Supplemental Figure S5C) and no examples of CP110 staining are shown. It might be wiser to focus a final model on their strongest data, the relationship between TTBK2 and CEP83.

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

The authors have made significant changes to this revised manuscript entitled " TTBK2 phosphorylates CEP83 in promoting cilia initiation ". In particular, they generated phospho-CEP83 antibodies, as well as an RPE1 knockout cell line of TTBK2 to address the major points, particularly the fact that the induction of endogenous CEP83 phosphorylation was mediated by TTBK2 upon ciliogenesis. In addition, they provided the required quantifications for immunofluorescence and Western blot experiments, as well as good quality blots, missing in the first version of the manuscript.
Overall, the new changes make the revised version appropriate for publication. However, some questions remain.

1-The immunolabelings with the phospho-CEP83 antibodies S29, T92 (Fig3G, H) show a cloudy staining between the 2 spots of gamma-tubulin, which is not the expected localization of CEP83 at the base of the cilium with a shape in ring. Could the authors explain why?
2- Why does re-expression of phospho-inactive CEP83 in RPE1 - / - cells not decrease ciliogenesis

compared with WT?
3- In Figure 7E, the authors wish to examine the effect of phosphorylation of CEP83 in establishing the transition zone. However, they should examine the presence of NPHP1 and TCNT2 at the TZ level exclusively in ciliated cells for cells expressing CEP834A.
5- The authors do not explain why CEP83 is so weakly expressed when co-expressed with TTBK2 (Fig. 3A). Can the TTBK2 control the stability of the CEP83?
6- The presentation of Fig.5 is a bit confusing. Could the authors try to provide a more logical organization?

POINT-by-POINT RESPONSES:

With regard to reviewer #2's points, we have gone through your responses and we have the following comments: As indicated in point#2ii by reviewer #2 (in the first round of review), CP110 removal is likely downstream of ciliary vesicle recruitment (PMID: 25686250). You should focus your discussion and Figure 7F and this linear relationship rather than discuss the less persuasive MMP9 story published last year.

We first thank the reviewer for the nice suggestion. Indeed, it is known that the removal of CP110 from the mother centrioles is a critical step for cilia initiation and CP110 removal occurs after docking of membrane vesicles to the mother centrioles (Lu et al., 2015; Schmidt et al., 2012; Tanos et al., 2013). Since the docking process is mediated by DAs and CEP83 is a protein located at DAs, our results suggest a model that CEP83 phosphorylation controls the docking of membrane vesicles the mother centrioles to promote CP110 removal and cilia initiation (Fig. 7H). We have modified our model, result, and discussion to state the linear relationship between membrane vesicle docking, CP110 removal, and cilia initiation.

In addition, you should use the term G0 rather than G1 when talking about serum-starved cells.

Sorry that we used G1 for the serum-starved cells in our manuscript. It has been corrected (figure legend in Fig. 6B). Thanks.

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

The authors have been very responsive to the comments of the reviewers. In particular, they carried out substantive new experiments that address important points.

They now show:

1) Using proteins purified from E. coli, they document the direct phosphorylation o CEP83 by TTBKs

2) They generated TTBK2-/- RPE1 cells and were able to show the CEP83 phosphorylation depends on TTBK2.

3) They made two phospho-specific antibodies against two of the four CEP83 sites (S29 and T292) and used vitro kinase assay and IF experiments to support the hypothesis that phosphorylation of each site is mediated by TTBK2.

The experiments provide convincing evidence that CEP83 is a bonafide TTBK2 target.

However, a weakness of the revised paper is that they do not address how significant their findings are for the regulation of cilia formation in vivo. For example, the authors do not carry out experiments that would compare the functions of two TTBK2 targets, CEP83 and CEP164.

We agreed with the reviewer that it is important to compare the functions of two TTBK2 targets, CEP83 and CEP164. Although the results obtained from the in vitro kinase show that CEP164 is phosphorylated by TTBK2 (Cajanek and Nigg, 2014), whether

CEP164 phosphorylation controls ciliogenesis is still not known. Thus, it is necessary to demonstrate that TTBK2-dependent CEP164 phosphorylation is important for ciliogenesis and know how it is regulated before we design experiments to analyze the relationship between TTBK2-dependent CEP83 and CEP164 phosphorylation.

In their final model (Figure 7H) they infer that phosphorylation of CEP83 by TTBK2 has two roles: removal of CP110 and ciliary vesicle docking. The data supporting this model are presented in two bar graphs (Fig 7F, G), with marginal significance (*). But the raw data showing an effect on ciliary vesicle association are limited to staining with Myosin Va (Supplemental Figure S5C) and no examples of CP110 staining are shown. It might be wiser to focus a final model on their strongest data, the relationship between TTBK2 and CEP83.

First, we've included the CP110 staining in our figure (Fig. 7C).

We conclude that CEP83 phosphorylation affects membrane vesicle docking and CP110 removal by performing the staining of CP110 and myosin-Va ***in both proliferating cells and serum-starved cells*** (Figs. 7C, 7D, 7F and 7G). Although we only observed marginal significance (*, between $CEP83^{WT}$ and $CEP83^{DEED}$) in proliferating cells (Figs. 7F and 7G), however, we saw dramatic significances (***, between $CEP83^{WT}$ and $CEP83^{4A}$) in serum-starved cells (Figs. 7C and 7D). It is known that ciliogenesis is a tightly regulated process that occurs only when cells are in G_0 or early G1 phase. In our figures 7F and 7G, we force CEP83 phosphorylation ($CEP83^{DEED}$) in proliferating cells that most of cells are not at the right cell-cycle stage for ciliogenesis (Figs. 7F and 7G). The significance is only marginal in Figs. 7F and 7G might due to that some factors required for ciliogenesis are not fully expressed or functional in proliferating cells.

We agree that we need to modify our model according to our observation and published paper (Lu et al., 2015; Schmidt et al., 2012; Tanos et al., 2013). We have modified our text and figure to explain the relationship between membrane vesicle docking, CP110 removal, and cilia initiation (Fig. 7 H).

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

The authors have made significant changes to this revised manuscript entitled " TTBK2 phosphorylates CEP83 in promoting cilia initiation ". In particular, they generated phospho-CEP83 antibodies, as well as an RPE1 knockout cell line of TTBK2 to address the major points, particularly the fact that the induction of endogenous CEP83 phosphorylation was mediated by TTBK2 upon ciliogenesis. In addition, they provided the required quantifications for immunofluorescence and Western blot experiments, as well as good quality blots, missing in the first version of the manuscript.
Overall, the new changes make the revised version appropriate for publication. However, some questions remain.

1-The immunolabelings with the phospho-CEP83 antibodies S29, T92 (Fig3G, H) show a

cloudy staining between the 2 spots of gamma-tubulin, which is not the expected localization of CEP83 at the base of the cilium with a shape in ring. Could the authors explain why?

We stained endogenous CEP83 with two phospho-CEP83 antibodies to analyze the localization of phosphorylated CEP83^{S29} and CEP83^{T292} signals (Fig. S2F). Our results clearly indicated that the phosphorylated CEP83^{S29} and CEP83^{T292} signals were co-localized with endogenous CEP83. The images showing in Figs. 3G and 3H are probably due to the angle of the centrioles since we did not see it in other figures (Fig. 4I, Fig. S2D, and Fig. S2F).

2- Why does re-expression of phospho-inactive CEP83 in RPE1 - / - cells not decrease ciliogenesis compared with WT?

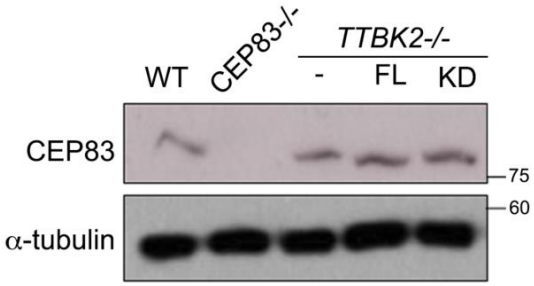
Based on the intensity quantification of phospho-CEP83 signals at the centrioles (Figs. 3G and 3H), it indicated that the level of CEP83 phosphorylation in proliferating cells is very low. Since ciliogenesis is a tightly regulated process that occurs only when cells are in G_0 or early G1 phase, the reason that we didn't see difference of ciliated frequency between CEP83^{WT} and CEP83^{4A} expressing cells is due to that most of cells are not in G_0 or early G1 phase (Fig. 6 D).

3- In Figure 7E, the authors wish to examine the effect of phosphorylation of CEP83 in establishing the transition zone. However, they should examine the presence of NPHP1 and TCNT2 at the TZ level exclusively in ciliated cells for cells expressing CEP834A.

We didn't quantify the intensity of NPHP1 and TCNT2 at the centrioles since we didn't observe intensity difference of TCTN2 and NPHP1 signals in ciliated cells in CEP83^{WT}, CEP83^{4A}, and CEP83^{DEED} expressing cells (Fig. 7E). Our results indicated that CEP83 phosphorylation promoted ciliary vesicle docking and CP110 removal (Figs. 7C and 7F). It is known that the ciliary vesicle docking and CP110 removal promoted transition zone assembly. Our results showed that CEP83 phosphorylation affected the recruitment of transition zone proteins TCTN2 and NPHP1, thus compromising the establishment of the transition zone.

5- The authors do not explain why CEP83 is so weakly expressed when co-expressed with TTBK2 (Fig. 3A). Can the TTBK2 control the stability of the CEP83?

We performed western blot of CEP83 in wild-type, *TTBK2-/-,* TTBK2^{FL}, and TTBK2^{KD} expressing cells to analyze the influence of TTBK2 in the control of CEP83 stability. Our result indicated neither TTBK2 overexpression nor inactivation affected the level of CEP83 (figure provided below). Since we co-transfected two plasmids (TTBK2 and CEP83) at the same time, the low transfection efficiency of CEP83 might be the reason to affect CEP83 expression (Fig. 3A).



6- The presentation of Fig.5 is a bit confusing. Could the authors try to provide a more logical organization?

Apologies for this. We've modified our text of figure 5 to make it in more logical organization. Here is how we modify our text.

Functional domain analysis of CEP83

We cloned and expressed various CEP83 mutants in *CEP83* knockout cells in order to map the region responsible for targeting CEP83 to DAs (Figs. 5 F and Fig. S4 E). The truncated forms of CEP83 carrying the C-terminal half of CEP83 ($CEP83^{151E}$ and $CEP83^{367E}$) were able to target to centrioles, whereas other fragments were not, indicating that CEP83 was recruited to centrioles via its C-terminus (Figs. 5, E and G; and Fig. S4 F). Given the role of CEP83 in DA assembly, we also mapped the region of CEP83 responsible for the recruitment of other DA proteins. Our results showed that CEP89 was recruited to the centrioles via the middle region of CEP83 (residues 151-366) (Figs. 5 H). The recruitment of CEP164 required both the N-terminal and middle regions of CEP83 since CEP164 was not detected at centrioles in $CEP83^{367E}$ expressing cells and was detected at centrioles in $CEP83^{151E}$ expressing cells, but at lower level compared to $CEP83^{WT}$ expressing cells (Fig. 5 I). The impaired recruitment of CEP164 to centrioles in $CEP83^{151E}$ expressing cells also reduced the association of TTBK2 with DAs and ciliogenesis (Figs. 5, J-L).

Reference:

Cajanek, L., and E.A. Nigg. 2014. Cep164 triggers ciliogenesis by recruiting Tau tubulin kinase 2 to the mother centriole. *Proceedings of the National Academy of Sciences of the United States of America*. 111:E2841-2850.

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Tanos, B.E., H.J. Yang, R. Soni, W.J. Wang, F.P. Macaluso, J.M. Asara, and M.F. Tsou. 2013. Centriole distal appendages promote membrane docking, leading to cilia initiation. *Genes Dev*. 27:163-168.

July 25, 2019

RE: JCB Manuscript #201811142RR

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Dear Prof. Wang:

Thank you for submitting your revised manuscript entitled "TTBK2 phosphorylates CEP83 in promoting cilia initiation". 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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