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## PLK2 phosphorylation is critical for CPAP function in procentriole formation during the centrosome cycle

Jaerak Chang, Onur Cizmecioglu, Ingrid Hoffmann, Kunsoo Rhee

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### Review timeline:

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### Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

28 October 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three expert reviewers, whose comments are copied below. As you will see, all of them find your results identifying Plk2 phosphorylation as regulatory mechanism for CPAP in centrosome duplication interesting and potentially important. At the same time, they however also raise a number of substantive points that currently appear to preclude publication in The EMBO Journal, at least in the present form. In this respect, some of the concerns raised have the potential to impinge on the main conclusions of the study, and their proper clarification would therefore be essential before any commitments towards publication could be made from our side. Nevertheless, given that the main criticism mostly refer to technical (rather than conceptual) problems, I am inclined to allow you the possibility to respond to the referees' comments and to address their points through a revised version of the manuscript. Should you be able to adequately address the major issues, we should be able to consider a revised manuscript for eventual publication. As it is EMBO Journal policy to allow a single round of major revision only, it will however be important to diligently answer to all the various experimental and editorial points raised at this stage if you wish the manuscript ultimately to be accepted. When preparing your letter of response, please also bear in mind that this will form part of the Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: <http://www.nature.com/emboj/about/process.html>). In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor  
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In this manuscript Chang and colleagues present exciting new data suggesting that CPAP is phosphorylated by PLK2 and that this phosphorylation is required at an early step during centriole duplication, more specifically during procentriole assembly. The data presented is novel, generally of high quality and provides much needed insights on the critical role played by phosphorylation in daughter centriole assembly. The authors should consider the following specific points:

- The data of the phosphorylation site mapping summarized in Fig 1A should be shown. It is important to show that full-length CPAP is phosphorylated *in vitro* and to control for the quality of the different CPAP truncations used in the kinase assays (this is only shown for GST-CPAP 563-613 in Fig 1B).

- The authors mention that their phospho-specific antibodies do not perform well in western blot. However, they do work in immunofluorescence assays. This is somewhat unfortunate since phospho-specific antibodies usually recognize linear epitopes, which should be detectable on Western blots. An alternative approach would be to use their phospho-specific antibody in immunoprecipitation experiments followed by blotting for total CPAP. This would provide *in vivo* biochemical evidence for CPAP phosphorylation and complement the immunofluorescence data (see next point).

- The specific localization of phosphorylated CPAP to procentrioles is an interesting observation. Considering the authors have used an approach allowing the use of two rabbit antibodies simultaneously one needs to be careful with the interpretation of the data based on these experiments alone mainly because of potentially incomplete labeling of one of the primary antibodies. Also intriguing is why the CPAP antibody does not overlap with the phospho-CPAP labelling and the authors should comment on that. A complementary approach using GFP-tagged CPAP, together with immuno-EM would further support their exciting observation. This is a major point of the paper which would greatly benefit from additional controls.

- The link between PLK2 and the localization of phospho-CPAP to procentrioles should be strengthened. If the model proposed by the authors is that PLK2 mediated phosphorylation of CPAP is required at an early step during procentriole assembly, a decrease in phospho-CPAP levels should be detected in PLK2 RNAi-treated cells.

- The authors show that CPAP-SSAA is unable to induce centriole elongation in contrast to WT CPAP and CPAP-SSEE. This result is consistent with the idea that the phosphorylation of CPAP by PLK2 at these residues is a prerequisite not only for centriole duplication, but as well for the assembly of these elongated centrioles. As discussed above, a nice control would be to show that the formation of these elongated structures upon overexpression of WT CPAP is perturbed under PLK2 RNAi conditions.

- The Coomassie gel of WT and KD kinase domain of PLK2 show additional bands in the WT forms. In addition, T563A and S592A mutants appear significantly less phosphorylated than WT. This should be commented upon.

- I am not convinced the PLK4 phosphorylation data presented adds significantly to the manuscript. Although the authors show that PLK4 can also phosphorylate CPAP at S595, they provide no evidence in support of a functional role, nor do they provide evidence that this site is actually phosphorylated *in vivo*. PLK4 is known to act before CPAP in daughter centriole assembly and it is therefore not surprising that the CPAP-SSEE mutation can't compensate for the loss of PLK4, which

likely has other yet unknown targets implicated in centriole duplication.

- The authors should show data on the depletion of endogenous PLK2. Only data with myc-tagged PLK2 is shown.
- The relative expression levels of endogenous and ectopic CPAP and mutant variants (SSAA, S595A and S589A) under the different experimental conditions should be explored. This would also allow the authors to comment of the relative stability of the proteins, which may differ.
- Two siRNA are used against CPAP, and as far as I can tell only Western blot analysis for CPAP-U is shown.
- The manuscript contains numerous typos and grammatical errors are frequent. It would certainly benefit from intense proofreading.

Referee #2 (Remarks to the Author):

In this manuscript, Jaerak Chang and Kunsoo Rhee present a novel study investigating an interesting link between PLK2 and CPAP in human cells, and how this link influences centriole biogenesis, in particular their length. The manuscript is very interesting, timely, and deserves to be published in EMBO, although some clarifications should be made that I will point out. The authors claim that:  
i PLK2 phosphorylates two residues in CPAP protein, S589 and S595 both in vitro and in vivo  
i that this phosphorylation is critical for CPAP function in centriole biogenesis and that it occurs in a cell cycle specific manner  
i S595 is also phosphorylated by PLK4, but this is not critical for procentriole assembly  
i CPAP phosphorylation increases at G1/S transition phase and decreases during M phase. The phosphorylated CPAP is preferentially located at the procentriole.

Major points:

- 1) "PLK2 phosphorylates two residues in CPAP protein, S589 and S595 both in vitro and in vivo". The in vitro part is well shown in Figure 1. However, it is not clear why the authors only use the kinase domain in their in vitro phosphorylation assays. Do they have same result with full kinase? (the polo box domain might be important in controlling substrate specificity and binding). Does PLK2 only phosphorylate one site in each CPAP molecule? Or do the authors see a 3rd band of different mobility when PLK2 phosphorylates both sites?
- 2) "that this phosphorylation is critical for CPAP function in centriole biogenesis"

A-Figure 1D- the authors say that the double mutant CPAP (SSAA) localizes to the centrosomes- this is difficult to see in the images as they are. It would be helpful to have bigger images and the myc channel should be zoomed in the centrosome in an inset. Figure 1D and 1E- Please indicate in figure legends how countings were made (definition of duplicated centrioles as 4 CP110 dots in 1D; vs 2 centrin dots in 1E ). In figure 1 H the authors say that almost 80% of the non transfected cells have duplicated centrioles. This is inconsistent with the FACS profile shown by these cells (Fig S3) where most cells appear to be in G1, so these cells should present 2 centrioles. Could the authors calculate the percentage of population in each cell cycle phase in Figure S3.

B-Figure 2- It would be important to refer to the cycle profile of PLK2 knockdown in the main text (Figure S3). Once more the population of cells in each cell cycle phase should be quantitated. In Figure 2C- The authors show that expression of the S589 mutant but not the single SE mutant is able to rescue PLK2 knock down. To be sure that each site on its own does not work as a dominant negative mutant, it would be important to test the effect of expressing the single and double mutant together with the siCTL.

- 3) "S595 is also phosphorylated by PLK4, but this is not critical for procentriole assembly"  
A-The authors show that one of the sites that is phosphorylated by PLK2 is also phosphorylated by PLK4. Authors should make a comment regarding why they use only the kinase domain of PLK4. The authors say in page 9 that:  
"Involvement of PLK4 phosphorylation at S595 of CPAP in procentriole assembly was examined by

the knockdown-rescue experiments. As reported previously, PLK4 suppression impaired the centriole duplication significantly (Figure 3C; Habadanck et al., 2005). However, neither wild type nor SSEE mutants of CPAP rescued the impaired centriole duplication in PLK4-depleted cells (Figure 3C). These results suggest that PLK4 phosphorylation at S595 of CPAP may not be a critical step for procentriole assembly. We do not agree with this interpretation- it could simply be that PLK4 has other functions, other substrates whose phosphorylation cannot be rescued with the CPAP phospho mimic mutant.

To fully understand the role of PLK2 and PLK4 phosphorylation on these two sites in vivo it would be very important to knock down independently and together, PLK2 and PLK4, in the context of a cell line expressing WT CPAP, and test with the phosphospecific antibodies which sites are phosphorylated by these kinases.

4) "CPAP phosphorylation increases at G1/S transition phase and decreases during M phase. The phosphorylated CPAP is preferentially located at the procentriole"

A-The authors generated phospho-specific antibodies against the different sites of CPAP phosphorylation. In page 10 the authors say that: "...we arrested the cell cycle at the G1/S transition phase by double thymidine block, when both PLK2 and PLK4 are in active states and measured centrosomal CPAP and phospho-CPAP levels (Fode et al., 1996; Warnke et al., 2004)." From the current literature, in particular the recent paper (Rogers et al, 2009) it seems that maximum levels of PLK4 might be attained in mitosis and that very little PLK4, if any, will be present in G1/S.

B- Interpretation of Figure 5- perhaps the most correct way to state those results would be "CPAP phosphorylation increases at G1/S transition phase and decreases during mitotic exit" rather than just "M phase" (Figure 5B). It would be interesting to be able to relate the amounts in Figure 5A and B. Do the levels of phosphorylated CPAP increase steadily from G1/S to metaphase? And to relate this with the different kinases- could it be that phosphorylation in G1/S is mediated by PLK2 and phosphorylation in mitosis is mediated by PLK4? This should be easy to test in synchronised cells, with knock downs of these proteins (single and double) and using the phospho-specific antibodies in immunostaining. The fact that two kinases are acting in the same site can point for a regulatory pathway, where one acts as a priming phosphorylation for the other.

C. In Figure 6- I find it too complicated the strategy that the authors use to show that phosphorylated CPAP is at the procentriole. Could the authors use c-NAP (intercentriole region) and centrin-GFP (distal part of the centriole) as markers to clarify the position of the procentriole? In duplicating centrioles the fainter centrin dot corresponds to the procentriole (Loncarek et al, 2008); if phosphorylated CPAP localises to the procentriole it should colocalise with the faint centrin-GFP dot and in between the cNAP dot and the centrin dot. What cell cycle phase were cells in this experiment?

The authors suggest in the discussion that CPAP may be stabilized by phosphorylation to participate in centriole assembly. Are the mutants they express in this manuscript more or less stable?

General considerations about the figures:

Very General- Panels showing immunofluorescence should be bigger, in all cases scale bars should be present as well as insets for the different channels. Reference for the zoom used in these insets should be present in the legend of the figure.

- Figure 1 is too big. The authors could consider breaking it in two (A, B and C separated from the rest).

- Figure 2 should be reorganised so that the immunostaining panels are bigger;

- In Figure 3, perhaps panels A and B could show only the relevant bands to save space.

- In Figure 4, the authors should explain in the legend how the intensity measurement was made: were the two centrioles measured at the same time? How is this influenced by the fact that centriole duplication is impaired in both PLK2 and PLK4 depletion?

- In Figure 5 In panel B, I find confusing why there is only one centriole marked with CPAP antibody, or does it mark only the daughter centriole? Authors should comment this.

-References are missing-

Page 3- During the exit from mitosis, the paired centrioles are disengaged by separase, which is considered a critical step for licensing centriole duplication (Tsou and Stearns, 2006a; Tsou and Stearns, 2006b). Misses-(Tsou et al, 2009)

Page 3- SPD-2 is responsible for the recruitment of centrosomal components, including ZYG-1

(Kemp et al., 2004). Misses-(Delattre et al, 2006; Pelletier et al, 2006; Pelletier et al, 2004)  
Page 4- There are no obvious homologues of ZYG-1 in the human genome, but PLK4 is speculated to be its functional homologue for initiation of centriole duplication (Kleylein-Sohn et al., 2007; Nigg, 2007). Misses-(Bettencourt-Dias et al, 2005)  
Page 4- As a human orthologue of SAS-4, importance of CPAP in procentriole assembly is evident (Kohlmaier et al., 2009). Misses-(Kleylein-Sohn et al, 2007; Tang et al, 2009)  
Page 9- Overexpression of PLK4 induces formation of multiple procentrioles from a single parental centriole (Habedanck et al., 2005; Kleylein-Sohn et al., 2007). Misses-(Cunha-Ferreira et al, 2009; Rogers et al, 2009)  
Page 14- PLK2 and PLK4 are known to have distinct structural properties especially at the polo domains and to have their own substrate specificities consequently (Leung et al., 2002; Elia et al., 2003). Misses-(Johnson et al, 2007)  
Page 14- Although centriole assembly is controlled by highly conserved mechanism throughout species, PLK2 is not conserved in invertebrates whereas PLK4 has an obvious structural and functional homologue in *Drosophila Melanogaster* (Peel et al., 2007). Misses-(Bettencourt-Dias et al, 2005)

Bettencourt-Dias M, Rodrigues-Martins A, Carpenter L, Riparbelli M, Lehmann L, Gatt MK, Carmo N, Balloux F, Callaini G, Glover DM (2005) SAK/PLK4 Is Required for Centriole Duplication and Flagella Development. *Curr Biol* 15: 2199-2207

Cunha-Ferreira I, Rodrigues-Martins A, Bento I, Riparbelli M, Zhang W, Laue E, Callaini G, Glover DM, Bettencourt-Dias M (2009) The SCF/Slimb ubiquitin ligase limits centrosome amplification through degradation of SAK/PLK4. *Curr Biol* 19(1): 43-49

Delattre M, Canard C, Gonczy P (2006) Sequential protein recruitment in *C. elegans* centriole formation. *Curr Biol* 16(18): 1844-1849

Johnson EF, Stewart KD, Woods KW, Giranda VL, Luo Y (2007) Pharmacological and functional comparison of the polo-like kinase family: insight into inhibitor and substrate specificity. *Biochemistry* 46(33): 9551-9563

Kleylein-Sohn J, Westendorf J, Le Clech M, Habedanck R, Stierhof YD, Nigg EA (2007) Plk4-induced centriole biogenesis in human cells. *Dev Cell* 13(2): 190-202

Pelletier L, O'Toole E, Schwager A, Hyman AA, Muller-Reichert T (2006) Centriole assembly in *Caenorhabditis elegans*. *Nature* 444(7119): 619-623

Pelletier L, Ozlu N, Hannak E, Cowan C, Habermann B, Ruer M, Muller-Reichert T, Hyman AA (2004) The *Caenorhabditis elegans* centrosomal protein SPD-2 is required for both pericentriolar material recruitment and centriole duplication. *Curr Biol* 14(10): 863-873

Rogers GC, Rusan NM, Roberts DM, Peifer M, Rogers SL (2009) The SCF Slimb ubiquitin ligase regulates Plk4/Sak levels to block centriole reduplication. *J Cell Biol* 184(2): 225-239

Tang CJ, Fu RH, Wu KS, Hsu WB, Tang TK (2009) CPAP is a cell-cycle regulated protein that controls centriole length. *Nat Cell Biol* 11(7): 825-831

Tsou MF, Wang WJ, George KA, Uryu K, Stearns T, Jallepalli PV (2009) Polo kinase and separase regulate the mitotic licensing of centriole duplication in human cells. *Dev Cell* 17(3): 344-354

Referee #3 (Remarks to the Author):

Centrosomal P4.1-Associated Protein (CPAP) is the human ortholog of SAS-4, a component of the centriole assembly machinery which is conserved from ciliates to vertebrates. SAS-4 acts at a late step in centriole assembly, the addition of the microtubule wall. Consistent with this function, CPAP has been shown to have both tubulin dimer and microtubule-binding activities *in vitro*, while overexpression of CPAP *in vivo* results in abnormal extensions of the centriole wall. In metazoans, centriole assembly is initiated by the polo-family kinase PLK4. Its distant relative, PLK2 has also

been implicated in this process in human cells, although the evidence for this is much weaker.

In the present manuscript, Chang and Rhee identify CPAP as a substrate for PLK2 (and PLK4) phosphorylation *in vitro*, which they map to two residues (S589, S595) in the putative microtubule-binding domain of the protein (amino acids 423-607; Hsu et al., *Exp. Cell Res.* 2008). Mutation of these residues to alanine results in a dominant negative effect on centrosome duplication and also prevents the formation of filamentous extensions of the centriolar wall observed in cells overexpressing wild-type CPAP. Using phospho-specific antibodies, the authors show that the phosphorylation status of these residues changes in a cell-cycle-dependent manner, with high levels from G1/S until early mitosis, during which time CPAP is thought to be active in promoting centriole assembly. While these observations are potentially interesting, as detailed below there are significant caveats to this work and some of the main conclusions are not supported by the data presented.

#### Main points

1. It is difficult to evaluate the *in vitro* kinase assay data as currently presented. For Fig. 1A, autoradiograms and coomassie gels should be shown as in subsequent panels. +/- is not the way to display this kind of data. With only 6 potential phosphorylation sites in the 50 amino acid fragment used from panels 1B onward, it is not surprising that 2 mutations eliminate phosphorylation. It would be more informative to see the effect in the context of larger fragments of CPAP, if not the full-length protein.
2. The residues identified lie within the previously established microtubule-binding domain of CPAP. Mutation of these residues to alanine may thus affect microtubule binding, which could explain the dominant negative phenotypes observed. The authors need to eliminate this possibility by performing microtubule pelleting assays with wild-type and alanine mutant CPAP fragments. Since bacterially expressed proteins are not phosphorylated, there should not be any difference in microtubule binding activity between wild-type and alanine mutant protein.
3. The evidence for PLK2 as a critical regulator of CPAP activity *in vivo* is weak. Unlike depletion of PLK4, depletion of PLK2 only marginally affects centrosome duplication (Fig. 2B,C). In this context, the 'rescue' by the phosphomimetic mutant is not meaningful. The absence of rescue with PLK4 (Fig. 3C) does not exclude PLK4 phosphorylation of S595 as a critical step in centriole assembly: Since PLK4 is required for upstream events in centriole assembly, a bypass of these events with a phosphomimetic CPAP mutant cannot be expected.
4. The feeble effect of depleting either kinase on phosphorylated S589 and S595 epitopes at centrosomes is puzzling. Shouldn't there be more of an effect on CPAP phosphorylation, if these kinases are the critical regulators of CPAP phosphorylation at these residues? Depletion of CPAP should be performed to confirm specificity of the phosphoantibodies and establish a baseline for these experiments. Further, immunoblots with phospho-S589 and S595 antibodies following PLK2/4 RNAi would provide a more quantitative measure of the relative contribution of each kinase.
5. The immunofluorescence pictures in Fig. 6 do not, in my view, show any clear difference in localization of phosphorylated vs total CPAP. This figure could be much reduced/eliminated.

1st Revision - authors' response

02 February 2010

Referee #1 (Remarks to the Author):

*In this manuscript Chang and colleagues present exciting new data suggesting that CPAP is phosphorylated by PLK2 and that this phosphorylation is required at an early step during centriole duplication, more specifically during procentriole assembly. The data presented is novel, generally of high quality and provides much needed insights on the critical role played by phosphorylation in daughter centriole assembly. The authors should consider the following specific points:*

*- The data of the phosphorylation site mapping summarized in Fig 1A should be shown. It is important to show that full-length CPAP is phosphorylated in vitro and to control for the quality of the different CPAP truncations used in the kinase assays (this is only shown for GST-CPAP 563-613 in Fig 1B).*

As suggested, the site mapping data were included in the revised manuscript (Supplementary Figure S1A, B, C and D). The kinase assays were carried out with diverse truncated GST-CPAP fusion proteins. (Intact forms of full-length GST-CPAP proteins could not be purified because of the huge size of 190 kDa.) PLK2 was prepared both from culture cells by immunoprecipitation as well as from bacteria by purification. The results collectively show that S589 and S595 of CPAP are the major phosphorylation sites for PLK2.

*- The authors mention that their phospho-specific antibodies do not perform well in western blot. However, they do work in immunofluorescence assays. This is somewhat unfortunate since phospho-specific antibodies usually recognize linear epitopes, which should be detectable on Western blots. An alternative approach would be to use their phospho-specific antibody in immunoprecipitation experiments followed by blotting for total CPAP. This would provide in vivo biochemical evidence for CPAP phosphorylation and complement the immunofluorescence data (see next point).*

We tried every possible ways to detect phospho-CPAP in immunoblots. We performed immunoprecipitation followed by immunoblot analysis and immunoblotting with the centrosome fractions, but we failed to detect the phospho-CPAP band. Adjusting immunoblot conditions did not help. It is likely that the phospho-CPAP antibodies detect an excess amount in immunoblot analysis. The data in Supplementary Figure S7A shows that our phospho-specific antibodies recognize phospho-CPAP proteins when 1 g protein of GST-CPAP563-613 was used as a substrate for in vitro kinase assay.

*- The specific localization of phosphorylated CPAP to procentrioles is an interesting observation. Considering the author have used an approach allowing the use of two rabbit antibodies simultaneously one needs to be careful with the interpretation of the data based on these experiments alone mainly because of potentially incomplete labeling of one of the primary antibodies.*

In order to avoid experimental artifacts, we used three marker antibodies (CPAP, CEP135 and - tubulin) which were independently conjugated with a fluorescent dye. We also performed the experiments with two different phospho-CPAP antibodies and observed identical staining patterns (data not shown).

*Also intriguing is why the CPAP antibody does not overlap with the phospho-CPAP labeling and the authors should comment on that.*

In Figure 6A, the phospho-CPAP labeling is merged with the CPAP labeling. The arrowheads indicate CPAP dots partially overlapped with phospho-CPAP dots.

*A complementary approach using GFP-tagged CPAP, together with immuno-EM would further support their exciting observation. This is a major point of the paper which would greatly benefit from additional controls.*

We are afraid that immune-EM experiments are currently not available for us. Instead, we observed procentriolar localization of phospho-CPAP by immunostaining culture cells (Fig. 6A), amplified centrioles (Fig. 6B), and elongated centrioles (Fig. 7C). We hope that the experimental evidence may be sufficient to conclude that the phospho-CPAP is preferentially localized at the procentriole.

*- The link between PLK2 and the localization of phospho-CPAP to procentrioles should be strengthened. If the model proposed by the authors is that PLK2 mediated phosphorylation of CPAP*

*is required at an early step during procentriole assembly, a decrease in phospho-CPAP levels should be detected in PLK2 RNAi-treated cells.*

In Figure 4 and Supplementary Figure S7C, we showed a reduction in phospho-CPAP levels in PLK2-depleted cells. We also observed that the PLK2-depletion blocked the CPAP-induced procentriole elongation but it did not block the CPAPSSEE-induced procentriole elongation (Figure 7B).

*- The authors show that CPAP-SSAA is unable to induce centriole elongation in contrast to WT CPAP and CPAP-SSEE. This result is consistent with the idea that the phosphorylation of CPAP by PLK2 at these residues is a prerequisite not only for centriole duplication, but as well for the assembly of these elongated centrioles. As discussed above, a nice control would be to show that the formation of these elongated structures upon overexpression of WT CPAP is perturbed under PLK2 RNAi conditions.*

We performed the proposed experiment supporting that the centriole elongation activity of CPAP is dependent on PLK2 phosphorylation (Figure 7B). The results show that the CPAP-mediated centriole elongation does not occur in PLK2-depleted cells. The phospho-mimetic CPAP mutant (Myc-CPAPSSEE) can induce the centriole elongation even in PLK2-depleted cells.

*- The Coomassie gel of WT and KD kinase domain of PLK2 show additional bands in the WT forms. In addition, T563A and S592A mutants appear significantly less phosphorylated than WT. This should be commented upon.*

It is likely that the additional bands in WT kinase domain of PLK2 are degraded fragments of the protein. In order to avoid possible mis-interpretations, we repeated in vitro kinase assays using full-length PLK2 (Supplementary Figure S1E). Relative autoradiogram intensities of CPAP563-613, T563A and CPAP563-613, S592A repeatedly looked reduced but showed doublet phosphorylation bands as in the wild type. On the other hand, CPAP563-613, S589A and CPAP563-613, S595A showed a single phosphorylation band. Therefore, we concluded that S589A and S595A are phosphorylation sites for PLK2. It is possible that T563A and S592A of CPAP indirectly affect the phosphorylation activity at S589 and S595 rather than directly phosphorylated by PLK2.

*- I am not convinced the PLK4 phosphorylation data presented adds significantly to the manuscript. Although the authors show that PLK4 can also phosphorylate CPAP at S595, they provide no evidence in support of a functional role, nor do they provide evidence that this site is actually phosphorylated in vivo. PLK4 is known to act before CPAP in daughter centriole assembly and it is therefore not surprising that the CPAP-SSEE mutation can't compensate for the loss of PLK4, which likely has other yet unknown targets implicated in centriole duplication.*

We initially performed PLK4 kinase assay to confirm that CPAP is uniquely phosphorylated by PLK2. To our surprise, however, CPAP was phosphorylated by PLK4 specifically. We observed a reduction of the centrosomal pS595 level in PLK4-suppressed cells, suggesting that S595 of CPAP is phosphorylated by PLK4 in vivo (Figure 4; Supplementary Figure S7C). We totally agree that PLK4 should have other targets implicated in centriole assembly. Nonetheless, we think that it is worth to describe PLK4 phosphorylation of CPAP for future investigation. In fact, our data do not rule out the possibility that PLK4 is critical for CPAP function in centriole elongation.

*- The authors should show data on the depletion of endogenous PLK2. Only data with myc-tagged PLK2 is shown.*

We obtained the PLK2 antibody from Dr. Ingrid Hoffman in a collaboration base. The reduction in endogenous PLK2 proteins is shown in Figure 2A and B.

*- The relative expression levels of endogenous and ectopic CPAP and mutant variants (SSAA, S595A and S589A) under the different experimental conditions should be explored. This would also allow the authors to comment of the relative stability of the proteins, which may differ.*



Immunoblot analyses consistently showed that the ectopic CPAP levels are by 10-fold higher than the endogenous levels (Attached figure). We did not observe a difference in stability of the mutant variants in comparison to the wild type CPAP (Attached figure). In order to limit possible complications with excess expression, we observed the phospho-mutant CPAP phenotypes in both overexpressing and knockdown-overexpressing cells (Figure 1D, E and H).

- Two siRNA are used against CPAP, and as far as I can tell only Western blot analysis for CPAP-U is shown.

Western blot analysis for siCPAP was included in Supplementary Figure S3A.

- The manuscript contains numerous typos and grammatical errors are frequent. It would certainly benefit from intense proofreading.

The revised manuscript was edited by a professional English editor.

Referee #2 (Remarks to the Author):

*In this manuscript, Jaerak Chang and Kunsoo Rhee present a novel study investigating an interesting link between PLK2 and CPAP in human cells, and how this link influences centriole biogenesis, in particular their length. The manuscript is very interesting, timely, and deserves to be published in EMBO, although some clarifications should be made that I will point out. The authors claim that:*

- PLK2 phosphorylates two residues in CPAP protein, S589 and S595 both in vitro and in vivo
- that this phosphorylation is critical for CPAP function in centriole biogenesis and that it occurs in a cell cycle specific manner
- S595 is also phosphorylated by PLK4, but this is not critical for procentriole assembly
- CPAP phosphorylation increases at G1/S transition phase and decreases during M phase. The phosphorylated CPAP is preferentially located at the procentriole.

*Major points:*

1) *"PLK2 phosphorylates two residues in CPAP protein, S589 and S595 both in vitro and in vivo". The in vitro part is well shown in Figure 1. However, it is not clear why the authors only use the kinase domain in their in vitro phosphorylation assays. Do they have same result with full kinase? (the polo box domain might be important in controlling substrate specificity and binding).*

The kinase domain-only PLK2 (GST-PLK2-kido) was easy to be prepared. As suggested, we performed in vitro kinase assays with a full-length PLK2 protein and observed an identical result (Supplementary Figure S1E).

*Does PLK2 only phosphorylate one site in each CPAP molecule? Or do the authors see a 3rd band of different mobility when PLK2 phosphorylates both sites?*

In vitro kinase assay did not produce a third band which may be generated by phosphorylation at both S589 and S595. It is likely that phosphorylation at S595 does not induce a mobility shift, because the S595-phosphorylated band was not up-shifted in comparison to the unphosphorylated CPAP band stained with coomassie blue (Figure 1B and C). Therefore, it is not possible to determine whether both serines are simultaneously phosphorylated in vitro by PLK2 or not. Nonetheless, the mutant experiments indicate that phosphorylations at both S589 and S595 are critical for CPAP functions (Figure 1E).

2) *"that this phosphorylation is critical for CPAP function in centriole biogenesis"*

*A-Figure 1D- the authors say that the double mutant CPAP (SSAA) localizes to the centrosomes- this is difficult to see in the images as they are. It would be helpful to have bigger images and the myc channel should be zoomed in the centrosome in an inset.*

As suggested, the centrosomes in Figure 1D are zoomed in insets.

*Figure 1D and 1E- Please indicate in figure legends how countings were made (definition of duplicated centrioles as 4 CP110 dots in 1D; vs 2 centrobins dots in 1E).*

We determined unduplicated centriole with 1 or 2 CP110 dot(s), and duplicated centrioles with 3 or 4 CP110 dots (Figure 1D). In Figure 1E, unduplicated centriole includes 0 or 1 centrobins dot while duplicated centrioles include 2 or 3 centrobins dots. These are indicated at the figure legends.

*In figure 1 H the authors say that almost 80% of the non transfected cells have duplicated centrioles. This is inconsistent with the FACS profile shown by these cells (Fig S3) where most cells appear to be in G1, so these cells should present 2 centrioles. Could the authors calculate the percentage of population in each cell cycle phase in Figure S3.*

Fact is that the cellular centriole number does not fully correlate with the cellular DNA contents. That is, the initial assembly of the procentriole occurs at late G1 phase. CP110 associates with procentrioles regardless of their elongation state (Kleylein-Sohn et al., 2007). In fact, most of G1/S phase arrested cells by double thymine block include four CP110 dots even if their procentrioles are short. In contrast, if asynchronous HeLa cells were stained with a centrin-2 antibody, only about a half of cells contained duplicated centrioles (our unpublished data; Azimzadeh et al., 2009; Hemeryk et al., 2009). It is because centrin-2 is localized at more proximal part of centrioles than CP110. For this reason, we believe that CP110 is a proper marker to determine whether centriole duplication was started or not.

*B-Figure 2- It would be important to refer to the cycle profile of PLK2 knockdown in the main text (Figure S3). Once more the population of cells in each cell cycle phase should be quantitated.*

As suggested, the cell cycle profile of the CPAP- and PLK2-depleted cells was determined (Supplementary Figure S5).

*In Figure 2C- The authors show that expression of the SSEE mutant but not the single SE mutant is able to rescue PLK2 knock down. To be sure that each site on its own does not work as a dominant negative mutant, it would be important to test the effect of expressing the single and double mutant together with the siCTL.*

The suggested experiments were performed and presented in Supplementary Figure S6. The results show that the single mutants did not affect centriole duplication at all, indicating that the impaired centriole duplication is not due to a dominant negative effect but due to a failure in the phenotype rescue of PLK2 depletion.

*3) "S595 is also phosphorylated by PLK4, but this is not critical for procentriole assembly"  
A-The authors show that one of the sites that is phosphorylated by PLK2 is also phosphorylated by PLK4. Authors should make a comment regarding why they use only the kinase domain of PLK4.*

Since full-length of PLK4 was highly degraded when it was purified in bacteria, we used the kinase domain of PLK4 for in vitro kinase assay. The size of kinase domain-contained form of PLK4 was decided according to a previous report (Leung et al., 2007).

*The authors say in page 9 that:*

*"Involvement of PLK4 phosphorylation at S595 of CPAP in procentriole assembly was examined by the knockdown-rescue experiments. As reported previously, PLK4 suppression impaired the centriole duplication significantly (Figure 3C; Habedanck et al., 2005). However, neither wild type nor SSEE mutants of CPAP rescued the impaired centriole duplication in PLK4-depleted cells (Figure 3C). These results suggest that PLK4 phosphorylation at S595 of CPAP may not be a critical step for procentriole assembly." We do not agree with this interpretation- it could simply be that PLK4 has other functions, other substrates whose phosphorylation cannot be rescued with the CPAP phospho mimic mutant.*

We totally agree with the reviewer's comments. We do not ignore the importance of PLK4 in

centriole assembly. The main conclusion of this experiment is that PLK4 should phosphorylate other substrates to complete centriole duplication, in addition to CPAP. In case of PLK2, however, CPAP phosphorylation is necessary and sufficient for completion of centriole duplication. We modified the text to avoid a possible misunderstanding on the matter.

*To fully understand the role of PLK2 and PLK4 phosphorylation on these two sites in vivo it would be very important to knock down independently and together, PLK2 and PLK4, in the context of a cell line expressing WT CPAP, and test with the phosphospecific antibodies which sites are phosphorylated by these kinases.*

We tried hard but realized that the cells die when both PLK2 and PLK4 were knocked down simultaneously. Therefore we knocked down PLK2 and PLK4 independently and observed that the phosphorylation levels at S589 were reduced in PLK2-depleted cells while those at S595 were reduced in both PLK2- and PLK4-depleted cells (Figure 4; Supplementary Figure S7C).

*4) "CPAP phosphorylation increases at G1/S transition phase and decreases during M phase. The phosphorylated CPAP is preferentially located at the procentriole"*

*A-The authors generated phospho-specific antibodies against the different sites of CPAP phosphorylation. In page 10 the authors say that: "...we arrested the cell cycle at the G1/S transition phase by double thymidine block, when both PLK2 and PLK4 are in active states and measured centrosomal CPAP and phospho-CPAP levels (Fode et al., 1996; Warnke et al., 2004)." From the current literature, in particular the recent paper (Rogers et al, 2009) it seems that maximum levels of PLK4 might be attained in mitosis and that very little PLK4, if any, will be present in G1/S.*

We initially decided to measure the CPAP phosphorylation at G1/S at which the centriole assembly occurred. In the revised manuscript, we measured the CPAP phosphorylation levels in M phase cells (Supplementary Figure S7C). The results are similar to Figure 4 in which phosphorylation of CPAP S595 was reduced in both PLK2- and PLK4-depleted cells, while phosphorylation of CPAP S589 was reduced in PLK2-depleted cells.

*B- Interpretation of Figure 5- perhaps the most correct way to state those results would be "CPAP phosphorylation increases at G1/S transition phase and decreases during mitotic exit" rather than just "M phase" (Figure 5B).*

We corrected the text as suggested.

*It would be interesting to be able to relate the amounts in Figure 5A and B. Do the levels of phosphorylated CPAP increase steadily from G1/S to metaphase? And to relate this with the different kinases- could it be that phosphorylation in G1/S is mediated by PLK2 and phosphorylation in mitosis is mediated by PLK4? This should be easy to test in synchronized cells, with knock downs of these proteins (single and double) and using the phospho-specific antibodies in immunostaining. The fact that two kinases are acting in the same site can point for a regulatory pathway, where one acts as a priming phosphorylation for the other.*

As mentioned above, we measured the CPAP phosphorylation levels in M phase cells (Supplementary Figure S7C). We observed that phosphorylation of CPAP S595 was reduced in both PLK2- and PLK4-depleted cells irrespective of the cell cycle stages. Phosphorylation of CPAP S589 was reduced only in PLK2-depleted cells at both G1/S and M phase cells. Double knockdown of PLK2 and PLK4 was not technically plausible. These results indicate that S595 is phosphorylated by PLK2 and PLK4 throughout the cell cycle. We believe that a series of experiments should be performed to determine biological meanings of PLK4 phosphorylation of CPAP S595.

*C. In Figure 6- I find it too complicated the strategy that the authors use to show that phosphorylated CPAP is at the procentriole. Could the authors use c-NAP (intercentriole region) and centrin-GFP (distal part of the centriole) as markers to clarify the position of the procentriole? In duplicating centrioles the fainter centrin dot corresponds to the procentriole (Loncarek et al, 2008); if phosphorylated CPAP localises to the procentriole it should colocalise with the faint*

*centrin-GFP dot and in between the cNAP dot and the centrin dot. What cell cycle phase were cells in this experiment?*

We agree with reviewer's opinion that the most appropriate method showing the localization of phospho-CPAP is triple staining with centrin and C-NAP1. We tried but we could not obtain a clear result of the triple immunostaining. We instead showed phospho-CPAP signals are partially merged with the CPAP dot, and merged with a faint CPAP dot which corresponds to the procentriole in G1/S phase cells (Figure 6A).

*The authors suggest in the discussion that CPAP may be stabilized by phosphorylation to participate in centriole assembly. Are the mutants they express in this manuscript more or less stable?*

Ectopic protein levels of CPAPWT, CPAPSSAA and CPAPSSEE were not different to each other and they were detected at the centrosome (see Figures 1D, E, H and 2D). Therefore, we believe that phosphorylation does not affect the protein stability as well as centrosomal localization. However, results in Figures 6 and 7 suggest that phosphorylation is required for procentriolar incorporation and centriolar elongation. Therefore, we propose that phospho-CPAP may be stably incorporated into procentrioles. We modified the text to avoid a possible misunderstanding on the matter.

*General considerations about the figures:*

*Very General- Panels showing immunofluorescence should be bigger, in all cases scale bars should be present as well as insets for the different channels. Reference for the zoom used in these insets should be present in the legend of the figure.*

We corrected the figures as suggested.

*- Figure 1 is too big. The authors could consider breaking it in two (A, B and C separated from the rest).*

We corrected the figures as suggested.

*- Figure 2 should be reorganised so that the immunostaining panels are bigger;*

We corrected the figures as suggested.

*- In Figure 3, perhaps panels A and B could show only the relevant bands to save space.*

We corrected the figures as suggested.

*- In Figure 4, the authors should explain in the legend how the intensity measurement was made: were the two centrioles measured at the same time? How is this influenced by the fact that centriole duplication is impaired in both PLK2 and PLK4 depletion?*

We measured two centrosomes at the same time because the centrosomes are closely placed at G1/S phase. The results showed that the centrosomal CPAP level was not obviously reduced in PLK2- and PLK4-depleted cells, suggesting that defects in centriole assembly do not affect the recruitment of CPAP at G1/S phase.

*- In Figure 5 In panel B, I find confusing why there is only one centriole marked with CPAP antibody, or does it mark only the daughter centriole? Authors should comment this.*

Since CPAP is localized to proximal ends of both parental and procentrioles, in general, mitotic spindle poles which contain an engaged pair of centrioles looks to contain one CPAP dot. Sometimes, however, two distinguishable CPAP dots were observed due to their proper configuration or disengagement between two centrioles (Supplementary Figure S8B).

*-References are missing-*

*Page 3- During the exit from mitosis, the paired centrioles are disengaged by separase, which is considered a critical step for licensing centriole duplication (Tsou and Stearns, 2006a; Tsou and Stearns, 2006b). Misses-(Tsou et al, 2009)*

Page 3- SPD-2 is responsible for the recruitment of centrosomal components, including ZYG-1 (Kemp et al., 2004). Misses-(Delattre et al, 2006; Pelletier et al, 2006; Pelletier et al, 2004)  
Page 4- There are no obvious homologues of ZYG-1 in the human genome, but PLK4 is speculated to be its functional homologue for initiation of centriole duplication (Kleylein-Sohn et al., 2007; Nigg, 2007). Misses-(Bettencourt-Dias et al, 2005)  
Page 4- As a human orthologue of SAS-4, importance of CPAP in procentriole assembly is evident (Kohlmaier et al., 2009). Misses-(Kleylein-Sohn et al, 2007; Tang et al, 2009)  
Page 9- Overexpression of PLK4 induces formation of multiple procentrioles from a single parental centriole (Habedanck et al., 2005; Kleylein-Sohn et al., 2007). Misses-(Cunha-Ferreira et al, 2009; Rogers et al, 2009)  
Page 14- PLK2 and PLK4 are known to have distinct structural properties especially at the polo domains and to have their own substrate specificities consequently (Leung et al., 2002; Elia et al., 2003). Misses-(Johnson et al, 2007)  
Page 14- Although centriole assembly is controlled by highly conserved mechanism throughout species, PLK2 is not conserved in invertebrates whereas PLK4 has an obvious structural and functional homologue in *Drosophila Melanogaster* (Peel et al., 2007). Misses-(Bettencourt-Dias et al, 2005)

Missed references were included in the revised manuscript.

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Tang CJ, Fu RH, Wu KS, Hsu WB, Tang TK (2009) CPAP is a cell-cycle regulated protein that controls centriole length. *Nat Cell Biol* 11(7): 825-831

Tsou MF, Wang WJ, George KA, Uryu K, Stearns T, Jallepalli PV (2009) Polo kinase and separase regulate the mitotic licensing of centriole duplication in human cells. *Dev Cell* 17(3): 344-354

Referee #3 (Remarks to the Author):

*Centrosomal P4.1-Associated Protein (CPAP) is the human ortholog of SAS-4, a component of the centriole assembly machinery which is conserved from ciliates to vertebrates. SAS-4 acts at a late step in centriole assembly, the addition of the microtubule wall. Consistent with this function, CPAP*

*has been shown to have both tubulin dimer and microtubule-binding activities in vitro, while overexpression of CPAP in vivo results in abnormal extensions of the centriole wall. In metazoans, centriole assembly is initiated by the polo-family kinase PLK4. Its distant relative, PLK2 has also been implicated in this process in human cells, although the evidence for this is much weaker.*

*In the present manuscript, Chang and Rhee identify CPAP as a substrate for PLK2 (and PLK4) phosphorylation in vitro, which they map to two residues (S589, S595) in the putative microtubule-binding domain of the protein (amino acids 423-607; Hsu et al., Exp. Cell Res. 2008). Mutation of these residues to alanine results in a dominant negative effect on centrosome duplication and also prevents the formation of filamentous extensions of the centriolar wall observed in cells overexpressing wild-type CPAP. Using phospho-specific antibodies, the authors show that the phosphorylation status of these residues changes in a cell-cycle-dependent manner, with high levels from G1/S until early mitosis, during which time CPAP is thought to be active in promoting centriole assembly. While these observations are potentially interesting, as detailed below there are significant caveats to this work and some of the main conclusions are not supported by the data presented.*

#### *Main points*

*1. It is difficult to evaluate the in vitro kinase assay data as currently presented. For Fig. 1A, autoradiograms and coomassie gels should be shown as in subsequent panels. +/- is not the way to display this kind of data. With only 6 potential phosphorylation sites in the 50 amino acid fragment used from panels 1B onward, it is not surprising that 2 mutations eliminate phosphorylation. It would be more informative to see the effect in the context of larger fragments of CPAP, if not the full-length protein.*

As suggested, we included the site mapping data in Supplementary Figure S1A, B, C and D. It was not possible to use a full-length CPAP of 190 kDa in size as a substrate. When we used CPAP563-667, SSAA (which is twice larger than CPAP563-613, SSAA) as a substrate, we observed that it was not phosphorylated by GST-PLK2-kido efficiently (Attached figure). In addition, we performed in vitro kinase assay with full-length PLK2 as an enzyme (Supplementary Figure S1E).

*2. The residues identified lie within the previously established microtubule-binding domain of CPAP. Mutation of these residues to alanine may thus affect microtubule binding, which could explain the dominant negative phenotypes observed. The authors need to eliminate this possibility by performing microtubule pelleting assays with wild-type and alanine mutant CPAP fragments. Since bacterially expressed proteins are not phosphorylated, there should not be any difference in microtubule binding activity between wild-type and alanine mutant protein.*

We performed the proposed microtubule sedimentation assays and reported in Supplementary Figure S2A. The results show that the SSAA mutation does not affect the microtubule binding activity of GST-CPAP563-613 in vitro. Furthermore, we observed that the SSAA mutant also associated with depolymerized or bundled microtubules like WT CPAP (Supplementary Figure S2B). These results suggest that the SSAA mutation does not affect the microtubule binding activity of CPAP.

*3. The evidence for PLK2 as a critical regulator of CPAP activity in vivo is weak. Unlike depletion of PLK4, depletion of PLK2 only marginally affects centrosome duplication (Fig. 2B,C). In this context, the 'rescue' by the phosphomimetic mutant is not meaningful. The absence of rescue with PLK4 (Fig. 3C) does not exclude PLK4 phosphorylation of S595 as a critical step in centriole assembly: Since PLK4 is required for upstream events in centriole assembly, a bypass of these events with a phosphomimetic CPAP mutant cannot be expected.*

Figure 3C indicates that PLK4 phosphorylation at S595 of CPAP is not sufficient for centriole duplication. It is likely that additional substrates should be phosphorylated by PLK4 for the centriole duplication. In fact, we modified the text to avoid a possible misunderstanding on the matter. Although the function of PLK2 in centriole duplication is regarded as less important than PLK4, PLK2-dependent regulation of the centriole duplication is definitely necessary because its suppression not only represses centriole overduplication in S phase arrested U2OS cells but also reduces the number of centrioles in asynchronous cells (Warnke et al., 2004; Cizmecioglu et al.,

2008; Figure 2). Therefore, we believe that the phospho-mimetic mutant rescued the part which is managed by PLK2 in centriole duplication.

*4. The feeble effect of depleting either kinase on phosphorylated S589 and S595 epitopes at centrosomes is puzzling. Shouldn't there be more of an effect on CPAP phosphorylation, if these kinases are the critical regulators of CPAP phosphorylation at these residues? Depletion of CPAP should be performed to confirm specificity of the phosphoantibodies and establish a baseline for these experiments.*

As suggested, we measured the phospho-CPAP levels at the centrosome in CPAP-depleted G1/S phase cells (Supplementary Figure S7B). When the centrosomal CPAP levels were depleted to 30% of the control levels, the centrosomal phospho-CPAP levels were reduced less than by 50%. This suggests that the baseline staining intensity of the phospho-CPAP signals are high enough to make the decreased level in PLK2-depleted cells significant (Fig. 4).

*Further, immunoblots with phospho-S589 and S595 antibodies following PLK2/4 RNAi would provide a more quantitative measure of the relative contribution of each kinase.*

We tried very hard to detect phospho-CPAP in immunoblots. We tried immunoprecipitation followed by immunoblot analysis and immunoblotting with the centrosome fractions, but we failed to detect the phospho-specific band. An increase in the amount of the lysates and adjusting immunoblot conditions did not help. We believe that the endogenous phosphorylation level of CPAP may not be sufficient to be detected by the phospho-antibodies in immunoblots. The data in Supplementary Figure S7A shows that our phospho-specific antibodies are able to recognize phospho-CPAP proteins when 1 µg protein of GST-CPAP563-613 was used as a substrate for in vitro kinase assay.

*5. The immunofluorescence pictures in Fig. 6 do not, in my view, show any clear difference in localization of phosphorylated vs total CPAP. This figure could be much reduced/eliminated.*

It is difficult to distinguish CPAP which is localized at the proximal ends of the mother centriole and procentriole. Immunostaining pattern of the CPAP antibody is clearly larger than those of the phospho-CPAP antibodies (Figure 6A). We believe that the immunostaining of the overduplicated centrioles of U2OS cells with phospho-CPAP suggest the procentriole localization of the phospho-CPAP (Figure 6B).

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Polo-like kinase-2 is required for centriole duplication in mammalian cells. *Curr Biol* 14(13): 1200-1207

2nd Editorial Decision

17 February 2010

Thank you for submitting your revised manuscript for consideration by The EMBO Journal, which has now been seen once more by the three original referees. On the whole, these referees appreciate that the study has been improved in various aspects in response to the original comments. However, it is also clear that significant concerns remain, and that the paper is therefore still not a good candidate for publication in The EMBO Journal. Some of these criticisms had already been present during the initial round of review and insufficiently addressed, others have arisen from the data added during revision, especially regarding the phosphosite mapping data in Figure S1 and the microtubule pelleting assays. While these data may not be most crucial for the conclusions of the study, the most important concerns remain regarding the use of antibodies to (phosphorylated) CPAP, which is central to the paper. Given the amount of revision work that has already gone into the study, I am inclined to allow you to respond once more to the referees' criticisms, and will thus grant you an exceptional additional round of revision. Pending adequate improvement of the manuscript and/or response to the main criticisms, we should be able to consider your manuscript further for publication. While some points like the microtubule assay are not essential for the success of this re-revision and could be taken out due to the alternative confirmation as indicated by referee 3, alleviating the lingering concerns regarding phosphor-CPAP at centrosomes will however be the key issue for eventual success of the final version of this manuscript. I am hoping you will be able to get a re-revised version of the manuscript including a response letter back to us as soon as possible. Of course, please feel free to contact me if there is anything to be clarified further before submission of this final revision.

Yours sincerely,

Editor  
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In their revised manuscript, Rhee and colleagues present a much improved manuscript. Several of the issues I raised have been addressed textually, with additional experiments or in the rebuttal. Many of the concerns from the other referees have also been addressed.

My lingering concerns are mostly related to the fact that their phospho-specific antibody do not work by Western blot and the strength of the data on the localization of phosphorylated CPAP to procentrioles. These two issues, in my opinion, have not been address in the revised version of the manuscript. I understand the difficulty associated with getting these experiments to work, especially concerning the Western blot problems they have had with the phospho-specific antibodies, and getting EM data. At a minimum without further data related conclusions should be toned down.

Referee #2 (Remarks to the Author):

In this manuscript, Jaerak Chang and colleagues present a novel study investigating an interesting link between PLK2 and CPAP in human cells, and how this link influences centriole biogenesis, in particular their length. The authors answered most of my concerns and have greatly improved the



manuscript, however I still have a few suggestions/questions.

1-In my considerations about the figures I had suggested that "...in all cases scale bars should be present as well as insets for the different channels." Very often the authors do not have insets for the CPAP channel in CPAP (WT and mutants) overexpression. This would help greatly as in some cases it is not clear how strong this signal is (for example Figure 1E, H).

2-The myc-CPAP signal is very weak (Figure 1D) and not very focused- very different from antibody staining (eg Figure 4). Any reason for this?

3-It is of some concern that when the authors knock down CPAP, the Phospho-CPAP signal is only reduced by 40-45%, in comparison to an overall reduction of 70% of CPAP (Figure S7B). Can the authors explain this result? The specificity of these phosphospecific antibodies is so crucial for the temporal resolution of the phosphorylation (Figure 6).

4-Regarding the sentence-

"These results suggest that PLK4 phosphorylation at S595 of CPAP may not be a critical step for procentriole assembly." We still do not agree with this interpretation- it could simply be that PLK4 has other functions, other substrates whose phosphorylation cannot be rescued with the CPAP phospho mimic mutant.

I would substitute for "These results suggest that PLK4 phosphorylation at S595 of CPAP is not a critical step in PLK4 role in procentriole assembly."

Referee #3 (Remarks to the Author):

In the revised version of their manuscript, the authors have sought to address the reviewers' concerns. However, significant weaknesses remain that impact the conclusions the authors wish to make, as detailed below.

Both Reviewer 1 and myself asked for the raw data for the mapping of the phosphorylation sites for PLK2 on CPAP presented in Fig. 1A, which is essential to assess the significance of the two phosphosites identified (S589, S595). This data is now shown in Supplemental Figure 1. There are several problems with this data: 1) There is no side-by-side comparison of overlapping fragments of CPAP, with different kinase preparations used on different fragments (N-terminus only with PLK2 immunoprecipitated from extracts, C-terminus only with bacterially expressed kinase domain). 2) The quality of several fragments is suspect (e.g. GST-CPAP 370-562 appears significantly smaller than 563-667 Fig S1C), suggesting significant degradation of the substrate material.

Since the putative phosphosites fall into the previously identified microtubule-binding domain of CPAP, I had asked for a microtubule pelleting assay to be performed on wild-type and alanine mutant CPAP to exclude the possibility of non-specific effects of the alanine mutation on microtubule binding (which would explain the failure to support centriole elongation in cells overexpressing this mutant). While the authors did carry out a sedimentation assay (Supplemental Fig 2A), and indeed some CPAP co-pellets with microtubules, they neglected to include important controls to rule out non-specific pelleting (either CPAP in the absence of tubulin, or addition of nocodazole to shift tubulin, and hopefully CPAP, into the supernatant - see Fig. 3c, Hsu et al., *Exp Cell Res* 2008). No conclusions can therefore be drawn from these experiments. More informative is the transfection experiment (Supplemental Fig 2B), which shows Alanine-mutant CPAP co-localizing with taxol-stabilized microtubules.

A significant limitation of this work is the inability of the authors to detect phosphorylated CPAP by immunoblotting with their phospho-specific antibodies, forcing them to rely on less quantitative immunofluorescence methods. In my previous comments, I queried the apparent weak effects of depleting PLK2/4 on phospho-CPAP. The authors have now included a critical control, depleting CPAP itself, which unfortunately shows an even weaker effect on phospho-CPAP levels at centrosomes (especially compared with total CPAP antibody). This rather illustrates the limitations of this approach, which makes it difficult to conclude much about the relative contributions of PLK2/4 on CPAP phosphorylation at these residues.

On a more positive note, the depletion of PLK2 in the context of overexpressed CPAP, as suggested by Reviewer 1, does support the authors' model of PLK2 phosphorylation promoting CPAP activity in centriole elongation, with PLK2 depletion suppressing centriole elongation by excess CPAP, indicating a requirement for PLK2 that is bypassed by mutating the two residues they identified to Glutamic Acid.

In conclusion, while key experiments (ruling out an effect of mutating the phosphosites on microtubule-binding and codepleting PLK2 while overexpressing CPAP) have strengthened the authors' model for a role for PLK2 in regulating CPAP activity at centrioles, the revised manuscript still suffers from significant shortcomings that affect critical aspects of this work.

2nd Revision - authors' response

09 May 2010

Referee #1 (Remarks to the Author):

*In their revised manuscript, Rhee and colleagues present a much improved manuscript. Several of the issues I raised have been addressed textually, with additional experiments or in the rebuttal. Many of the concerns from the other referees have also been addressed.*

*My lingering concerns are mostly related to the fact that their phospho-specific antibodies do not work by Western blot and the strength of the data on the localization of phosphorylated CPAP to procentrioles. These two issues, in my opinion, have not been addressed in the revised version of the manuscript. I understand the difficulty associated with getting these experiments to work, especially concerning the Western blot problems they have had with the phospho-specific antibodies, and getting EM data. At a minimum without further data related conclusions should be toned down.*

As mentioned in the previous response, our phospho-antibodies could not detect the endogenous CPAP protein in immunoblot analysis with whole cell lysates. Therefore, we tried immunoblot analysis with the immunoprecipitated endogenous CPAP proteins. The results showed that both pS589CPAP and pS595CPAP antibodies detected specific bands in control cells but not in CPAP-depleted cells (Figure 4B). Furthermore, the pS589CPAP-specific band was diminished in PLK2-depleted cells (Figure 4B). The pS595CPAP-specific band intensity was reduced in both PLK2- and PLK4-depleted cells (Figure 4B). This observation is consistent with the conclusion that S595 is phosphorylated by both PLK2 and PLK4.

We also detected the phospho-CPAP-specific bands in cells overexpressing ectopic Myc-CPAP proteins (Figure 4C). The intensity of both phospho-CPAP-specific bands was reduced significantly in PLK2-depleted cells (Figure 4C). These results confirmed that our phospho-antibodies detect the phospho-CPAP proteins specifically. Furthermore, the results revealed that S589 is phosphorylated by PLK2 and S595 is phosphorylated by both PLK2 and PLK4 in vivo.

In order to determine the exact localization of the phospho-CPAP proteins within a centriole pair, we immunostained the centrioles with three different antibodies simultaneously. We used CEP135 (red) and centrin (blue) antibodies to mark the proximal end of parental centriole and procentriole, respectively (Figure 6B). As expected, The CPAP signal (green) was largely overlapped with the CEP135 signal at the proximal region of the parental centriole (Figure 6B, upper panel). On the other hand, the phospho-CPAP proteins (green) were localized between the CEP135 and centrin signals (Figure 6B, middle and lower panels). We also obtained a similar result in double labeling of the centriole pairs with the CEP135 and centrin antibodies (Supplementary Figure S9). These results are consistent with the conclusion that the phospho-CPAP proteins are predominantly localized at the proximal region of the procentriole.

Referee #2 (Remarks to the Author):

*In this manuscript, Jaerak Chang and colleagues present a novel study investigating an interesting link between PLK2 and CPAP in human cells, and how this link influences centriole biogenesis, in particular their length. The authors answered most of my concerns and have greatly improved the*

manuscript. However I still have a few suggestions/questions.

1-In my considerations about the figures I had suggested that "...in all cases scale bars should be present as well as insets for the different channels." Very often the authors do not have insets for the CPAP channel in CPAP (WT and mutants) overexpression. This would help greatly as in some cases it is not clear how strong this signal is (for example Figure 1E, H).

As suggested, the scale bars were presented in all immunostaining figures. We also added insets for the Myc (CPAP) channels and the merged channels in CPAP overexpression.

2-The myc-CPAP signal is very weak (Figure 1D) and not very focused- very different from antibody staining (eg Figure 4). Any reason for this?

We obtained the pictures in Figure 1D and H of the previous version when we were inexperienced in immunostaining and photographing. In this revised version of the manuscript, we repeated the same experiment and replaced Figure 1D and H with new pictures.

3-It is of some concern that when the authors knock down CPAP, the Phospho-CPAP signal is only reduced by 40-45%, in comparison to an overall reduction of 70% of CPAP (Figure S7B). Can the authors explain this result? The specificity of these phosphospecific antibodies is so crucial for the temporal resolution of the phosphorylation (Figure 6).

In the revised manuscript, we included immunoblot results which confirmed the specificity of the phospho-CPAP antibodies (Figure 4B). Furthermore, PLK2 and PLK4 depletion effectively reduced the phospho-specific CPAP band intensities (Figure 4B, C). These results diminished the possibility that the phospho-CPAP antibodies detect a non-specific protein at the centrosome of the CPAP-depleted cells. It is not clear why the centrosomal levels of phospho-CPAP are not reduced as much as those of CPAP did. It is possible that PLK2 and PLK4 may phosphorylate a residual CPAP, which may be preferentially concentrated at the centrosome. Nonetheless, these phosphorylations should not be sufficient for the biological activity of CPAP during procentriole assembly.

4-Regarding the sentence-

"These results suggest that PLK4 phosphorylation at S595 of CPAP may not be a critical step for procentriole assembly." We still do not agree with this interpretation- it could simply be that PLK4 has other functions, other substrates whose phosphorylation cannot be rescued with the CPAP phospho mimic mutant.

I would substitute for "These results suggest that PLK4 phosphorylation at S595 of CPAP is not a critical step in PLK4 role in procentriole assembly."

We appreciate for a proper comment. We replaced the sentence in the abstract and results sections.

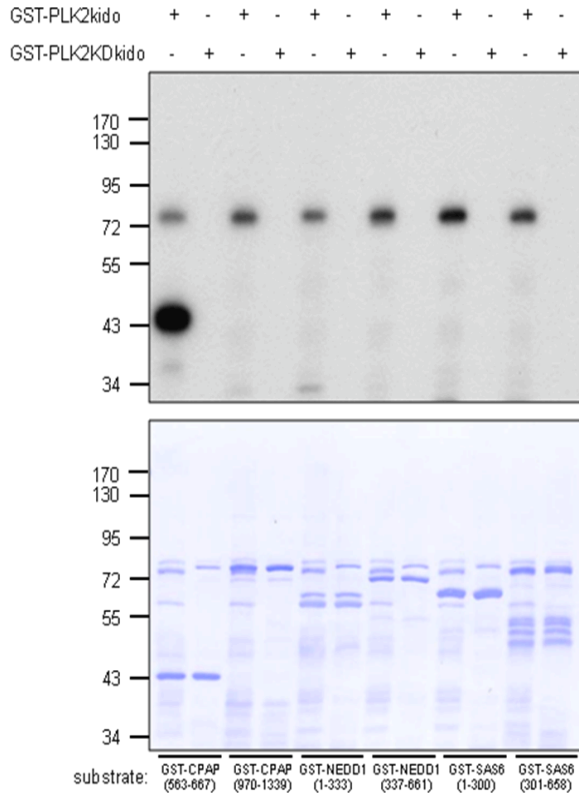
Referee #3 (Remarks to the Author):

In the revised version of their manuscript, the authors have sought to address the reviewers' concerns. However, significant weaknesses remain that impact the conclusions the authors wish to make, as detailed below.

Both Reviewer 1 and myself asked for the raw data for the mapping of the phosphorylation sites for PLK2 on CPAP presented in Fig. 1A, which is essential to assess the significance of the two phosphosites identified (S589, S595). This data is now shown in Supplemental Figure 1. There are several problems with this data: 1) There is no side-by-side comparison of overlapping fragments of CPAP, with different kinase preparations used on different fragments (N-terminus only with PLK2 immunoprecipitated from extracts, C-terminus only with bacterially expressed kinase domain). 2) The quality of several fragments is suspect (e.g. GST-CPAP 370-562 appears significantly smaller than 563-667 Fig S1C), suggesting significant degradation of the substrate material.

As soon as we learned that GST-CPAP is phosphorylated by PLK2 in vitro, we initiated the PLK2 kinase assays with GST-CPAP truncated mutants which are listed in Figure 1A. At the same

time, we screened additional substrates of PLK2 with GST-CPAP truncated mutant proteins as positive and negative controls. The attached figure is an example in which GST-CPAP<sup>563-667</sup> and GST-CPAP<sup>970-1339</sup> were used as controls for PLK2 kinase assays with GST-NEDD1 and GST-HsSAS-6 as substrates. From multiple PLK2 kinase assays, we knew that GST-CPAP<sup>563-613</sup> is the minimal fragment to be phosphorylated by GST-PLK2<sup>kido</sup>.



When we determined that S589 and S595 of CPAP are phosphorylation sites for PLK2, we used bacterially expressed N-terminus (including the kinase domain) of PLK2 (GST-PLK2kido) as an enzyme. GST-PLK2kido was useful since it is easy to be prepared and has a stable enzyme activity. After determining the specific phosphorylation sites, we decided to confirm the results with a full-length PLK2. Immunoprecipitated PLK2 phosphorylated GST-CPAP335-667 most predominantly (Supplementary Figure S1A). Bacterially expressed PLK2 did not phosphorylate GST-CPAPSSAA (Supplementary Figure S1E).

As the referee pointed out, we did not present a side-by-side comparison of overlapping fragments of CPAP with different kinase preparations used on different fragments. Nonetheless, we have performed the PLK2 kinase assays multiple times with all the CPAP fragments. Specific phosphorylation activity was confirmed with the full-length PLK2 proteins purified from bacteria and immunoprecipitation. We do not rule out the possibility that PLK2 may phosphorylate CPAP other than S589 and S595. In fact, it is a limitation of the *in vitro* kinase assay to define phosphorylation sites within a substrate. Perhaps mass spectrometric analysis may reveal additional phosphorylation sites within CPAP. However, our results with SSAA and SSEE mutants indicate that S589 and S595 are the phosphorylation sites for the biological activity of CPAP in centriole assembly (Figures 1-3). Furthermore, PLK2 phosphorylation at these sites was confirmed with the pS589CPAP and pS595CPAP antibodies (Figure 4). Therefore, we concluded that PLK2 phosphorylation at S589 and S595 is critical for centriole assembly.

We are embarrassed to confess that we made a stupid mistake in describing the size of a GST-CPAP truncated mutant. The correct sizes of the pointed mutants are GST-CPAP335-469 and GST-CPAP470-562 rather than GST-CPAP335-369 and GST-CPAP370-562. The expected size according to the corrected amino acid sequence is consistent with the actual size on SDS-PAGE (Supplementary Figure S1C). Therefore, we corrected Figure 1A, Supplementary Figure S1C and

their figure legends in the re-revised manuscript. We apologize for the confusion and appreciate for a careful correction.

*Since the putative phosphosites fall into the previously identified microtubule-binding domain of CPAP, I had asked for a microtubule pelleting assay to be performed on wild-type and alanine mutant CPAP to exclude the possibility of non-specific effects of the alanine mutation on microtubule binding (which would explain the failure to support centriole elongation in cells overexpressing this mutant). While the authors did carry out a sedimentation assay (Supplemental Fig 2A), and indeed some CPAP co-pellets with microtubules, they neglected to include important controls to rule out non-specific pelleting (either CPAP in the absence of tubulin, or addition of nocodazole to shift tubulin, and hopefully CPAP, into the supernatant - see Fig. 3c, Hsu et al., Exp Cell Res 2008). No conclusions can therefore be drawn from these experiments. More informative is the transfection experiment (Supplemental Fig 2B), which shows Alanine-mutant CPAP co-localizing with taxol-stabilized microtubules.*

We admit that the important controls are absent in Supplementary Figure S2A. However, we have already known that GST-CPAP563-613 does not precipitate for itself. In fact, GST-CPAP563-613 is one of the substrate for PLK2 kinase assay and it is soluble. Nonetheless, it would be much better if proper controls are included in Supplementary Figure S2A. As the referee pointed out, we did not observe any difference in subcellular distribution of Myc-CPAP and Myc-CPAPSSAA. This strongly suggests that alanine substitution did not affect the CPAP interaction activity with microtubules.

*A significant limitation of this work is the inability of the authors to detect phosphorylated CPAP by immunoblotting with their phospho-specific antibodies, forcing them to rely on less quantitative immunofluorescence methods.*

[The same response to Referee #1] As mentioned in the previous response, our phospho-antibodies could not detect the endogenous CPAP protein in immunoblot analysis with whole cell lysates. Therefore, we tried immunoblot analysis with the immunoprecipitated endogenous CPAP proteins. The results showed that both pS589CPAP and pS595CPAP antibodies detected specific bands in control cells but not in CPAP-depleted cells (Figure 4B). Furthermore, the pS589CPAP-specific band was diminished in PLK2-depleted cells (Figure 4B). The pS595CPAP-specific band intensity was reduced in both PLK2- and PLK4-depleted cells (Figure 4B). This observation is consistent with the conclusion that S595 is phosphorylated by both PLK2 and PLK4.

We also detected the phospho-CPAP-specific bands in cells overexpressing ectopic Myc-CPAP proteins (Figure 4C). The intensity of both phospho-CPAP-specific bands was reduced significantly in PLK2-depleted cells (Figure 4C). These results confirmed that our phospho-antibodies detect the phospho-CPAP proteins specifically. Furthermore, the results revealed that S589 is phosphorylated by PLK2 and S595 is phosphorylated by both PLK2 and PLK4 in vivo.

*In my previous comments, I queried the apparent weak effects of depleting PLK2/4 on phospho-CPAP. The authors have now included a critical control, depleting CPAP itself, which unfortunately shows an even weaker effect on phospho-CPAP levels at centrosomes (especially compared with total CPAP antibody). This rather illustrates the limitations of this approach, which makes it difficult to conclude much about the relative contributions of PLK2/4 on CPAP phosphorylation at these residues. On a more positive note, the depletion of PLK2 in the context of overexpressed CPAP, as suggested by Reviewer 1, does support the authors' model of PLK2 phosphorylation promoting CPAP activity in centriole elongation, with PLK2 depletion suppressing centriole elongation by excess CPAP, indicating a requirement for PLK2 that is bypassed by mutating the two residues they identified to Glutamic Acid.*

The immunoblot in Figure 4B of the revised manuscript confirmed that the phospho-antibodies recognize the phospho-CPAP proteins quite specifically. Furthermore, PLK2 and PLK4 depletion effectively reduced the phospho-specific CPAP band intensities (Figure 4B, C). These results diminished the possibility that the phospho-CPAP antibodies detect a non-specific protein at the centrosome of the CPAP-depleted cells. It is not clear why the centrosomal levels of phospho-CPAP are not reduced as much as those of CPAP did. It is possible that PLK2 and PLK4 may phosphorylate a residual CPAP, which may be preferentially concentrated at the centrosome. We still cannot rule out the possibility that there exist other kinases for phosphorylation at S589 and

S595. Nonetheless, these phosphorylations should not be sufficient to induce biological activity of CPAP for centriole assembly. Furthermore, as the referee pointed out, we observed that phosphorylation at S589 and S595 is critical for centriole elongation (Figure 7B). Taken together, we believe that S589 and S595 of CPAP should be sufficiently phosphorylated for its biological activity during procentriole assembly.

*In conclusion, while key experiments (ruling out an effect of mutating the phosphosites on microtubule-binding and codepleting PLK2 while overexpressing CPAP) have strengthened the authors' model for a role for PLK2 in regulating CPAP activity at centrioles, the revised manuscript still suffers from significant shortcomings that affect critical aspects of this work.*

In the revised manuscript, we confirmed the specificity of the phospho-CPAP antibodies. In addition, careful immunostaining data indicate that the phospho-CPAP proteins are localized at the proximal end of the procentriole. We believe that this information critically helps to convince the conclusion that specific phosphorylation at S589 and S595 of CPAP is critical for its biological activity during centriole assembly.

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Acceptance letter

12 May 2010

Thank you for submitting your revised manuscript for our consideration. I have now had a chance to carefully consider your responses and additional data, and to also discuss them with one of the original referees. As a result of these considerations, I am happy to inform you that we will now be able to accept this re-revised version for publication in The EMBO Journal.

You shall receive a formal letter of acceptance shortly.

Yours sincerely,

Editor  
The EMBO Journal