EMBO reports

Manuscript EMBOR-2011-35655

# Tankyrase 1 regulates centrosome function by controlling CPAP stability

Mi Kyung Kim, Charles Dudognon and Susan Smith

*Corresponding author: Susan Smith, The Skirball Institute*

**Review timeline:**

| | |
|---|---|
| Submission date: | 12 December 2011 |
| Editorial Decision: | 16 January 2012 |
| Revision received: | 04 April 2012 |
| Editorial Decision: | 24 April 2012 |
| Revision received: | 18 May 2012 |
| Accepted: | 23 May 2012 |

## 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 16 January 2012

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although all the referees find the topic of interest, referees 1 and 2 consider the study preliminary for publication here at this stage. Both of them consider that further experiments are needed to make the study conclusive, and also request a number of technical improvements of the data.

Given that all referees provide constructive suggestions on how to make the work more conclusive, I would like to give you the opportunity to revise your manuscript. As the referee reports are detailed below, I will not repeat them here. In this case, we consider important to address all points brought up by referees 1 and 2, including the use of a more specific or site-directed CPAP mutant to assay its interaction with tankyrase 1 and its stability, the demonstration of CPAP PARsylation by tankyrase 1 in vivo, the analysis of CPAP PARsylation and ubiquitination, and strengthening the quantification and statistical analyses throughout the study, as well as including some requested controls and improving the quality of some images.

If the referee concerns can be adequately addressed, we would consider your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, do not hesitate to get in touch with me if I can be of any assistance.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

1. Do the contents of this manuscript report a single key finding? YES

In this manuscript the authors demonstrate that the degradation and function of CPAP is regulated by the poly(ADP-ribose) polymerase tankyrase 1. The authors provide evidences to show that tankyrase 1 binds CPAP through a conserved RXXPDG tankyrase-binding motif located at its C terminus and this binding may result in the PARsylation of CPAP and regulates CPAP stability. They further demonstrate that CPAP can be PARsylated by tankyrase 1 in vitro. Depletion of tankyrase 1 by siRNA treatment induces an increase in CPAP protein and overexpression of tankyrase 1 leads to loss of CPAP and inhibits centriole duplication. The authors conclude that tankyrase 1-mediated PARsylation regulates CPAP levels to ensure normal centriole duplication during the cell cycle.

2. Is the main message supported by compelling experimental evidence? YES

Overall, this manuscript provides convincing data for most of the arguments and conclusions made, however, more experimental data described below are needed to support this conclusion.
1). Using an in vitro PARP assay, the authors clearly demonstrate that the recombinant GST-CPAP-C can be PARsylated by tankyrase 1 in vitro (Fig. 2). Whether CPAP is PARsylated by tankyrase 1 in vivo needs to be clarified.
2). Several proteins including TRF1, axin1, axin2, and tankyrase 1 itself has been demonstrated to be PARsylated by tankyrase 1 and subsequently led to their ubiquitylation and proteasome degradation. The authors should examine the status of both PARsylation and ubiquitylation of CPAP in the same cells.
3). The authors should provide statistical data with standard derivation in Fig. 3 and Fig. 4.

3. Have similar findings been reported elsewhere (e.g. on a closely related protein; in another organism or context)? NO

Tankyrase 1-mediated PARsylation has been reported to affect the degradation of several proteins including TRF1, axin1, axin2, and tankyrase itself. But none of them were reported to be involved in the centrosome function as CPAP did here.

4. Is the main finding of general interest to molecular biologists? YES

CPAP is a key player that participates in centriole duplication and mutations in CPAP gene cause primary microcephaly in humans. The major finding of this manuscript describes the control mechanism that regulates CPAP degradation and is suitable for molecular biologists of EMBO reports.

5. After appropriate revision, would a resubmitted manuscript be most suited for publication:

in EMBO reports

Referee #2:

In the manuscript "Tankyrase 1 regulates CPAP stability and centrosome function", Kim and colleagues study the regulation of CPAP levels, an important molecule involved in microcephaly and centriole formation. The authors claim that CPAP degradation and function is controlled by the poly(ADP-ribose) polymerase tankyrase 1, an important molecule involved in DNA damage. Being true this is the first link between tankyrase and centriole biogenesis, which is very interesting; however I feel that the experiments were not performed in the best way and that several controls are missing, therefore this manuscript is too preliminary.

The authors claim that experimentally:
1-CPAP interacts with tankyrase in vivo (Figure 1)
2-Overexpression of tankyrase 1 leads to proteasomal degradation of CPAP and prevented centriole duplication. (Figure 1; Figure 3)
3-CPAP is PARsylated by tankyrase 1 in vitro. (Figure 2)
4-Tankyrase 1 localizes to centrosomes exclusively in G1 coinciding precisely with the timing of CPAP degradation. (Figure 3, Figure 5)
5-Depletion of tankyrase 1 leads to stabilization of CPAP in G1 and elongated procentrioles and multipolarity. (Figure 4, Figure 5)

Major Comments:

Claim 1-
Figure 1-Tankyrase interacts with CPAP through the G-box- this is a huge deletion- this domain is very important for CPAP function. Since the authors know what is the interaction domain (conserved sequence- 1B), why didn´t they just delete that small sequence (6 residues versus 270)? This would be critical for many experiments in the manuscript as it would be much more specific than removing the whole G-box.

Claim 2-
Figure 1-Overexpression of tankyrase does indeed lead to reduced CPAP levels. Could this be due to changes in the cell cycle due to increased tankyrase expression? One alternative would be to do this experiment by expressing CPAP mutated in the conserved tankyrase-recognition box (6 aa) and ask whether it is more stable. Have the authors seen more ubiquitylated CPAP when overexpressing tankyrase?
Flag CPAP level are reduced by the OE of TNKS1 (1C) but this result is not shown in the OE of flagTNKS1 and MycCPAP. One control is missing: Myc CPAP alone to compare the level of myc-CPAP, when TNKS is OE or not in the input. 1F western blot should be quantified as it is not clear whether TNKS.PD has effect on flag-CPAP stability. 1G western blot should also be quantified.
Figure 3-It should be clarified that doxycyline represses tankyrase expression vs inducing expression (more common). Insets of centrioles should be shown in all images. 3E- Would be important to use another centriole marker to validate that single dots of CP110 do not just reflect differential CP110 regulation. Centrin would be an adequate marker. The authors should also state whether they observed any multipolar spindles, as poles with single centrioles could also result from premature centriole splitting with consequent multipolar formation. Are the cells that have less centrioles the ones that have less CPAP?

Claim 3- CPAP is PARsylated by tankyrase1 in vitro (figure2)
Figure 2-Inhibition of TNKS1: there is still some parsylation of CPAP in vitro but not anymore TNKS1 autoparsylation. What is the explanation for this? Does the Mutation of the TNKS1 binding domain prevent CPAP PARsylation?

Claim 4-
Figure 5- Why only 16% of the cells in G1 show tankyrase?

Claim 5-
Figure 4-In order to show that depletion of tankyrase leads to elongated centrioles, the authors should quantify centriole length using a proximal and distal centriole marker (eg-SAS6 and CP110) or a proxi for centriole length (centrin dot size- not as good). A control should be done with overexpression of CPAP. The effect the authors see is minor (5%), is this significant at all? Insets

should be shown for centriole regions. In order to show that depletion of tankyrase leads to an increase in centriole number, which is due to an increase in CPAP and not cell cycle changes or aborted cytokinesis, the authors should show that cell cycle progression is normal and also use another centriole marker. Ideally the authors should mutate or delete CPAP-tankyrase binding domain (Figure 1B) and show that in that case there is an increase in CPAP levels, centriole length and centriole number. As such the result would be much more direct.
- Figure 4C: the nuclei look very different in size: two different scales? Centrioles do not seem bigger.
- Figure 4G: The Quality of the images is poor rendering it very difficult to count the number of centrioles.

Figure 5-To show that tankyrase is specifically inducing the degradation of CPAP in G1 the authors should quantify the levels of CPAP and cyclin B on the western they are showing (normalized with tubulin levels) for at least two experiments. I find it strange that cyclin B comes up in the next time point. And if there is no accumulation of CPAP in S and G2, how do the authors explain the effect in centriole elongation? (It is at that stage that CPAP is supposed to promote elongation).
- Fig 5B: centrioles do not seem to be longer

Discussion-Was not clear for me why the authors think tankyrase could have come in the clustering screen- perhaps if depletion of tankyrase leads to supernumerary centrosomes, then clustering can be less efficient (more centrosomes to cluster?).
How do the authors integrate tankyrase regulation of CPAP levels with CPAP regulation by the APC/Cdh1? Do they think they are complementary?

Minor Comments-

Figure 1-
- In the text, the authors say that U2OS cells were transfected with Flag CPAP and TNKS, but in the legend of the figure they say that 293T cells were transfected...
-Figure 4E,G- why do the authors use Sh11, while in the western they show sh13?

Referee #3:

CPAP/SAS4 is an important centrosomal protein required for centriole assembly. Mutations in this protein is responsible for autosomal recessive primary microcephaly. The mechanism by which this protein particpates in the building of centrioles is not yet understood. Its overexpression promotes aberrant centriole elongation, and this protein has been shown to possess a tubulin-binding domain having a sequestering activity for the tubulin dimer, suggesting a direct role in the stability of the centriole wall.
This report brings original results on the modification of the protein which add new twists in the control of its degradation during cell cyle progression. Identifying a tankyrase 1 (TNKS1) binding motif in CPAP, which is common to all substrates of this poly(ADP-ribose) polymerase, this reports demonstrates that TNKS1 PARsylates CPAP and regulates CPAP protein stability and function at centrosomes across the cell cycle. It should be of interest for the centrosome field.

The biochemical demonstration of the interaction between the two proteins, of the effect on the level of CPAP of co-transfecting the two proteins, and of the PARsylation of CPAP by TNKS1 are all convincing.

Inducible TNKS1 expression or inducible TNKS1-13 shRNA in HTC75 stable cell lines were used to demonstrate a direct effec on the level of endogenous CPAP with the expected consequences on the centrosome.

The last piece of data uses cell synchronisation in TNKS1-depleted cells to show that TNKS1 is specifically required for degradation of CPAP in G1 phase. And that it is enriched at the centrosomes in G1, when CPAP degradation is taking place.

Specific comments

The conclusion section is slightly surprising as it focuses on the possible role of TNKS1 in centrosome clustering, an issue which is not addressed in the paper, instead of discussing more directly relevant questions.
For example, how is TNKS1addressed to the centrosome in G1 ? Is it through its binding to CPAP ? Is CPAP the only centrosomal protein containing the TKNS1 binding motif ?
Also, a brief PubMed survey using 'PARP and Centrosome' shows several papers which could have been quoted and discussed here. PARP1 and PARP3 have been reported at the centrosome. Would it mean that PARsylation is a more general modification for centrosomal proteins ?

1st Revision - authors' response 04 April 2012

We are submitting a revised manuscript. We have responded to the referees' concerns with additional data and discussion. In particular, we: 1) generated a site directed CPAP mutant (CPAP.AA) and used it to demonstrate the specificity of CPAP stability and interaction with tankyrase 1; 2) demonstrated that CPAP was PARsylated and ubiquitylated in vivo; 3) strengthened the quantification and statistical analysis throughout the study; and 4) improved the quality of some images.

We provide a point-by-point response below to the referee's concerns.

## Response to Referee #1

*Referee #1 comments:*
*1). Using an in vitro PARP assay, the authors clearly demonstrate that the recombinant GST-CPAP-C can be PARsylated by tankyrase 1 in vitro (Fig. 2). Whether CPAP is PARsylated by tankyrase 1 in vivo needs to be clarified.*

We now show that CPAP is PARsylated in vivo by tankyrase 1 in a new Fig. 2D. CPAP was transfected into 293T cells, immunoprecipitated, and detected with anti-PAR antibody. We show that CPAP.WT (but not CPAP.AA; a new double point mutation that abrogates tankyrase 1 binding) is detected by anti-PAR antibody.

*2). Several proteins including TRF1, axin1, axin2, and tankyrase 1 itself has been demonstrated to be PARsylated by tankyrase 1 and subsequently led to their ubiquitylation and proteasome degradation. The authors should examine the status of both PARsylation and ubiquitylation of CPAP in the same cells.*

We now show that CPAP in ubiquitylated in vivo under the same conditions and in the same cells as we show for PARsylation in vivo. In new Fig. 2E and F, CPAP was transfected into 293T cells along with HA-ubiquitin, immunoprecipitated, and detected with anti-HA antibody.
*3). The authors provide statistical data with standard derivation in Fig. 3 and Fig. 4.*

We now provide statistical data with standard deviation in Fig. 3 and Fig. 4.

## Response to Referee #2

*Referee #2 comments:*
*Claim 1-*
*Figure 1-Tankyrase interacts with CPAP through the G-box- this is a huge deletion- this domain is very important for CPAP function. Since the authors know what is the interaction domain (conserved sequence- 1B), why didn´t they just delete that small sequence (6 residues versus 270)? This would be critical for many experiments in the manuscript as it would be much more specific than removing the whole G-box.*

We have now generated at double point mutation CPAP.AA (new Fig. 2A). We show that CPAP.AA does not bind TNKS1 (new Fig. 2B), is not degraded by tankyrase 1 (new Fig. 2C), and is not PARsylated in vivo (new Fig. 2D).

Claim 2-
*Figure 1-Overexpression of tankyrase does indeed lead to reduced CPAP levels. Could this be due to changes in the cell cycle due to increased tankyrase expression?*

We see loss of CPAP even with a short transient transfection of low levels of tankyrase 1 (see Fig. 1 E for example) – under these conditions we do not see cell cycle changes.

*One alternative would be to do this experiment by expressing CPAP mutated in the conserved tankyrase-recognition box (6 aa) and ask whether it is more stable.*

We do see some stabilization of CPAP.AA over CPAP.WT (Figs. 2C, compare lanes 2 and 4).

*Have the authors seen more ubiquitylated CPAP when overexpressing tankyrase?*

This experiment is difficult to perform (and interpret) since overexpression of tankyrase 1 leads to loss of CPAP (shown in Figs. 1C and 2C)

*Flag CPAP level are reduced by the OE of TNKS1 (1C) but this result is not shown in the OE of flagTNKS1 and MycCPAP. One control is missing: Myc CPAP alone to compare the level of myc-CPAP, when TNKS is OE or not in the input.*

The purpose of the experiment with MycCPAP (Fig. 1D) was to show co-IP of MycCPAP with TNKS1 dependent on the MycCPAP C-terminal domain –that is why we do not show MycCPAP alone. The experiment in Fig. 1F does show that overexpression of TNKS1 leads to loss of MycCPAP (lanes 2 and 3). This is also now shown in new Fig. 2C).

*1F western blot should be quantified as it is not clear whether TNKS.PD has effect on flag-CPAP stability.*

We have quantified the blot in 1F. The quantification shows that wild type TNKS1 (but not TNKS1.PD) reduces CPAP protein levels.

*1G western blot should also be quantified*

We quantified the blot in 1G (which is now Fig. 2G in the revised manuscript). The quantification shows that TNKS1 induces degradation of CPAP and this degradation is rescued by protease inhibitor.

*Figure 3-It should be clarified that doxycyline represses tankyrase expression vs inducing expression (more common).*

To avoid confusion, we now indicate it as -/+ induction in the Figure, Figure Legend, and Results. We explain that dox represses expression in the Materials and Methods section.

*Insets of centrioles should be shown in all images.*

We have added insets of centrioles for all images.

*3E- Would be important to use another centriole marker to validate that single dots of CP110 do not just reflect differential CP110 regulation. Centrin would be an adequate marker.*

We specifically obtained the CP110 antibody to used as a marker since it was demonstrated in the literature that CPAP siRNA leads to single CP110 dots at mitosis [(Chang et al (2010) EMBO J 29(14)2395-2406]. We therefore think it is the best marker for this experiment.

*The authors should also state whether they observed any multipolar spindles, as poles with single centrioles could also result from premature centriole splitting with consequent multipolar formation. Are the cells that have less centrioles the ones that have less CPAP?*

We did not detect multipolar spindles or a direct correlation between single centrioles and loss of CPAP.

*Claim 3- CPAP is PARsylated by tankyrase1 in vitro (figure2)*
*Figure 2-Inhibition of TNKS1: there is still some parsylation of CPAP in vitro but not anymore TNKS1 autoparsylation. What is the explanation for this?*

We agree with the reviewer TNKS1 PARsylation appears to be more sensitive to inhibition than CPAP PARsylation. We repeated the analysis at higher concentrations of inhibitors and show complete inhibition of TNKS1 and CPAP PARsylation (new Fig. 1I). We now state in the Methods that at lower

concentrations of inhibitors, tankyrase 1 autoPARsylation was fully inhibited, but some residual CPAP PARsylation remained.

*Does the Mutation of the TNKS1 binding domain prevent CPAP PARsylation?*

We now show that CPAP lacking the TNKS1 binding domain (CPAP-N) does not get PARsylated by TNKS1 in vitro (new Fig. 1H) and that the CPAP.AA mutant is not PARsylated in vivo (new Fig. 2D).

*Claim 4-*
*Figure 5- Why only 16% of the cells in G1 show tankyrase?*

Our analysis indicates that tankyrase 1 localizes to centrosomes in a narrow window of the cell cycle in early G1 hence only a fraction of the cells (even at that time point) show tankyrase 1staining.

*Claim 5-*
*Figure 4-In order to show that depletion of tankyrase leads to elongated centrioles, the authors should quantify centriole length using a proximal and distal centriole marker (eg-SAS6 and CP110) or a proxi for centriole length (centrin dot size- not as good).*

We have added new data (new Fig. 4E) quantifying centriole length by measuring the lengths of centrioles labeled with acetylated tubulin as was done by [Tang et al 2009 Nature Cell biology 11(7):825] who showed that centrioles lengthened upon CPAP overexpression.

*A control should be done with overexpression of CPAP. The effect the authors see is minor (5%), is this significant at all?*

We think 5% is significant since we do not detect elongated centrioles in control cells. We feel that overexpression of CPAP, (which has been already been described by Tang et al, 2009 Nature Cell biology 11(7):825) and Kohlmaier et al, 2009 Current Biology 19:1012, is not an appropriate control, since in our experiments we are not overexpressing CPAP, but rather, we are inducing a pulse of CPAP in a window of the cell cycle by preventing tankyrase 1 from going to the centriole at that time. While we clearly demonstrate an effect at centrioles, we do not expect it to be as robust as when CPAP is highly overexpressed in all cells across the cell cycle.

*Insets should be shown for centriole regions.*

We now provide insets for 4E (now Fig. 4F).

*In order to show that depletion of tankyrase leads to an increase in centriole number, which is due to an increase in CPAP and not cell cycle changes or aborted*

*cytokinesis, the authors should show that cell cycle progression is normal and also use another centriole marker.*

We show that cell cycle progression is normal (identical) between tankyrase shRNA and GFP control shRNA cell lines in Fig. 5A using FACS analysis and immunoblotting with the cell cycle markers cyclin B and Cdc20. We used centrin as a marker for multiple centrioles as this is the standard used throughout the literature for this assay.

*Ideally the authors should mutate or delete CPAP-tankyrase binding domain (Figure 1B) and show that in that case there is an increase in CPAP levels, centriole length and centriole number. As such the result would be much more direct.*

The focus of our manuscript is to show the impact of tankyrase 1 on CPAP expression and function. We feel that our demonstration that knockdown of tankyrase 1 regulates stabilization of CPAP precisely in the narrow window of the cell cycle where it is normally degraded and to the exact level at which it is expressed throughout the rest of the cell cycle, clearly shows that tankyrase 1 regulates CPAP levels. While it is always elegant to pursue a knockout-replacement strategy, we feel it is not required here to illustrate the effect of tankyrase 1 on CPAP.

*- Figure 4C: the nuclei look very different in size: two different scales? Centrioles do not seem bigger.*

The magnification is the same. The HTC75 cell line (an HT1080 human fibrosarcoma derivative) exhibits a wide range of nuclear sizes.

*- Figure 4G: The Quality of the images is poor rendering it very difficult to count the number of centrioles.*

We replaced the images in Fig. 4G (now Fig. 4H) with sharper images. However, we point out that the purpose of this figure is to illustrate multipolarity. Thus, we want to show all the poles in 1 frame. In order to do so, we have to forgo fine resolution at each pole. We have not analyzed the number of centrioles at each pole in cells with multipolar spindles and are not trying to illustrate that with Fig. 4G (now Fig. 4H).

We do provide high quality images that permit counting of the centrioles in Fig. 3E and Fig. 4F (for example) where the purpose is to illustrate centriole number.

*Figure 5-To show that tankyrase is specifically inducing the degradation of CPAP in G1 the authors should quantify the levels of CPAP and cyclin B on the western they are showing (normalized with tubulin levels) for at least two experiments.*

We now present quantification for the levels of CPAP and cyclin B across the cell cycle in Fig. 5A. We repeated this quantification for a second experiment and this is provided at the end of this rebuttal for the reviewer. The repeat analysis

supports the results in Fig. 5A; CPAP is not degraded in G1 in the absence of tankyrase 1. We point out to the reviewer that the cell cycle stage is shown (not only by cyclin B and Cdc20 immunoblot), but in addition by FACS analysis, which clearly indicates the 12 hr time point as G1.

*I find it strange that cyclin B comes up in the next time point.*

Our quantification indicates that Cyclin B is reduced at 12 hr and then begins to return (although not to its fullest levels) in the next time point 14, consistent with cell cycle progression into S phase.

*And if there is no accumulation of CPAP in S and G2, how do the authors explain the effect in centriole elongation? (It is at that stage that CPAP is supposed to promote elongation).*

In the control cells we observe a gradual increase in CPAP through S phase up to mitosis as previously reported. Then there is a 5-fold reduction at G1. In the absence of tankyrase 1 we see that CPAP is high at the 0 time (G1/S) and is present throughout the cell cycle. We suggest that the inability to degrade CPAP upon exit from mitosis and during entry into G1 promotes continued elongation of the centriole.

*- Fig 5B: centrioles do not seem to be longer*

The purpose of Fig 5B was not to show longer centrioles (that is provided in Fig. 4A-D and new Fig. 4E), but rather, to show that endogenous tankyrase 1 goes to centrioles in G1 phase of the cell cycle coinciding with the time when CPAP is degraded.

*Discussion-Was not clear for me why the authors think tankyrase could have come in the clustering screen- perhaps if depletion of tankyrase leads to supernumerary centrosomes, then clustering can be less efficient (more centrosomes to cluster?).*

We do not know why tankyrase 1came in the clustering screen – we just know that it was reported in the literature [Kwon et al, (2008) G&D 22:2189]. However, we have eliminated the clustering screen from the discussion. Reviewer 3 pointed out that since we do not do any experiments to address clustering, rather that talk about clustering why not discuss other more related areas of interest, which is what we have done in our new discussion.

*How do the authors integrate tankyrase regulation of CPAP levels with CPAP regulation by the APC/Cdh1? Do they think they are complementary?*

They may be complementary. It is possible that the APC/C-Cdh1 system recognizes PARsylated CPAP.

*Minor Comments-*

*Figure 1-*
*- In the text, the authors say that U2OS cells were transfected with Flag CPAP and TNKS, but in the legend of the figure they say that 293T cells were transfected...*

It is 293T cells. We have corrected it in the text.

*-Figure 4E,G- why do the authors use Sh11, while in the western they show sh13?*

We showed previously that tankyrase 1 is knocked down to the same extent in shTNKS1-11, 12, 13 [Smith and Hsiao (2009) J Cell Biol 184(4):515]. We selected TNKS1-11 for the images in Fig. E (now Fig. 4F) and G (now Fig. 4H), but the analysis and quantification was performed with all three cell lines and gave similar results (Fig. 4G and I).

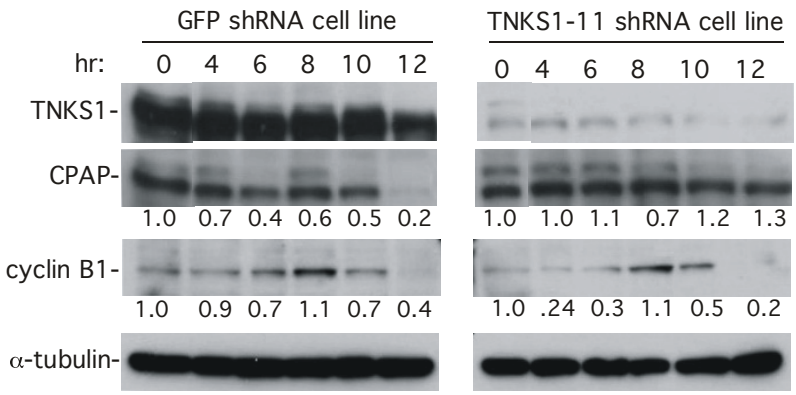
## Response to Referee #3

*Referee #3:*
*Specific comments*
*The conclusion section is slightly surprising as it focuses on the possible role of TNKS1 in centrosome clustering, an issue which is not addressed in the paper, instead of discussing more directly relevant questions.*
*For example, how is TNKS1addressed to the centrosome in G1 ? Is it through its binding to CPAP ? Is CPAP the only centrosomal protein containing the TKNS1 binding motif ?*
*Also, a brief PubMed survey using 'PARP and Centrosome' shows several papers which could have been quoted and discussed here. PARP1 and PARP3 have been reported at the centrosome. Would it mean that PARsylation is a more general modification for centrosomal proteins ?*

We agree with the reviewer and very much appreciate the suggestions. We have a new discussion where we address the issues suggested.

REPEAT OF CELL CYCLE ANALYSIS FOR REFEREE #2.




Immunoblot analysis of staged cell cycle extracts from GFP (left panel) or TNKS1-11 (right panel) stable HTC75 shRNA cell lines. Cells were synchronized by a double thymidine block and released for 0 hr (G1/S), 4 hr (mid S), 6 hr (late S), 8 hr (G2), 10 hr (M), and 12 hr (early G1), and 14 hr (mid G1). Protein levels relative to alpha-tubulin and normalized to the 0 hr time point are indicated beneath the blots.

The results show that in the control GFP cell line, CPAP is degraded at the12 hr (early G1) time point concomitant with cyclin B degradation. In the TNKS1 depleted cell line CPAP is not degraded in G1, while cyclin B is degraded as in control. The results are in agreement with the cell cycle analysis in Fig. 5A showing that tankyrase 1 is required for CPAP degradation in G1.

2nd Editorial Decision 24 April 2012

Thank you for your patience while your revised study was under peer-review. We have now received the enclosed reports from referees 1 and 2 of your initial version. As you will see, although all acknowledge the effort made during revision and the improvements provided, referee 2 has some remaining concerns of issues that were not appropriately addressed.

Upon further discussion with the referees, referee 1 also stated that centrin is a more suitable centriolar marker than CP110 and it is advisable to have more than one marker. In addition, s/he felt the concern voiced regarding figure 4 is also valid and would be addressable by increase the counting number of elongated centrioles using appropriate markers to resolve whether they are elongated or supernumenary centrioles.

EMBO reports normally only allows one round of revision, but as the outstanding issues seem addressable in a straightforward manner, I have decided to open an exceptional second round of revision. If these two concerns can be adequately addressed, we would be happy to consider your manuscript for publication.

I have also noticed that in figures 3F and 4D you have calculated SD from two independent experiments, which is incorrect (for guidance, please refer to for guidance: Cumming et al. JCB 2007). In the final manuscript, I would suggest to present the data from one experiment, clearly stating in the legend that this is a representative experiment of two conducted. Alternatively, you

could increase the number of independent experiments to three to perform statistical analysis.

Do let me know if I can be of any further assistance.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

The authors have successfully addressed the majority of my concerns in their revised manuscript. The improved version is suitable for publication in EMBO Reports.

Referee #2:

In the manuscript "Tankyrase 1 regulates CPAP stability and centrosome function", Kim and colleagues study the regulation of CPAP levels, an important molecule involved in microcephaly and centriole formation.

In the revised version the authors have addressed the comments regarding the in vitro and biochemical assays, however I still have many doubts regarding the in vivo, centriole data. This is very important regarding the biological outcomes described here and therefore I think this manuscript still needs major revision.

Figure 3- As the authors say, CP110 is used as a centriole marker; however this is a highly regulated molecule, whose loss could reflect its regulation, rather than centriole loss. To show loss of centrioles would be important to use another centriole marker such as centrin (short of doing electron microscopy, which would be much more laborious). Many centriolar molecules are highly regulated (even centrin), that is why most authors start to use more than one marker to validate their findings.

Figure 4- The authors have used acetylated tubulin as a proxy for centriole length, which is potentially good. However, in the pictures they show as examples for elongated centrioles, they rather seem to have supernumerary centrioles than long ones. The elongated centrioles shown in figures 4 C and E look like three distinct centrioles. The quality of the images is also low and perhaps makes it more difficult to appreciate whether there are longer centrioles present. Perhaps using a proximal and a distal marker would make it easier to quantitate, or centrin intensity, as we have suggested before.
Regarding figure 4H, it would be better to score numbers with two centriolar markers, instead of just one, because centrin can also form aggregates.

This data would also gain tremendously if the authors could express their CPAP-AA mutant that does not bind tankyrase, and compare its cellular phenotype to the expression of CPAP WT version. This would indeed be a more direct proof that tankyrase regulates directly CPAP levels and centriole function.

Figure 5-FACS profile could not be seen in my manuscript version.

2nd Revision - authors' response 18 May 2012

We are submitting our revised manuscript. We have addresses the three issues raised in your letter:

1. We provide a new Fig. 3G and H, where we performed the analysis of scoring cells with single centrioles using centrin antibody. We obtained similar results as with the CP110 antibody (Fig. 3E and F), thereby confirming that the result is not specific to CP110.

2. We provide a new Fig. 4F, G, and H, where we measure elongated centrioles using centrin antibody. The elongated centriole appears as a thread that can clearly be distinguished from supernumerary centrioles (see inset in Fig. 4F right panel). To measure the frequency of elongated centrioles we have performed three independent experiments (Fig. 4F and G) and obtained similar results as with anti-CP110 (shown in Fig. 4C and D). For measurement of the length we measured 40 centrioles (Fig. 4H) and obtained similar results as with anti-CP110 (shown in Fig. 4E).

3. We have revised Fig. 3F and 4D. We removed the error bars and now instead show the data for the two independent experiments in the graphs.

Response to Refee#1

*Referee #1comments:*
*The authors have successfully addressed the majority of my concerns in their revised manuscript. The improved version is suitable for publication in EMBO Reports.*

Thanks!

Response to Refee#2

*Referee #2 comments:*

*In the manuscript "Tankyrase 1 regulates CPAP stability and centrosome function", Kim and colleagues study the regulation of CPAP levels, an important molecule involved in microcephaly and centriole formation.*

*In the revised version the authors have addressed the comments regarding the in vitro and biochemical assays, however I still have many doubts regarding the in vivo, centriole data. This is very important regarding the biological outcomes described here and therefore I think this manuscript still needs major revision.*

*Figure 3- As the authors say, CP110 is used as a centriole marker; however this is a highly regulated molecule, whose loss could reflect its regulation, rather than centriole loss. To show loss of centrioles would be important to use another centriole marker such as centrin (short of doing electron microscopy, which would be much more laborious). Many centriolar molecules are highly regulated (even centrin), that is why most authors start to use more than one marker to validate their findings.*

We provide a new Fig. 3G and H, where we performed the analysis of scoring cells with single centrioles using centrin antibody. We obtained similar results as with the CP110 antibody in Fig. 3E and F), thereby confirming that the result is not specific to CP110.

*Figure 4- The authors have used acetylated tubulin as a proxy for centriole length, which is potentially good. However, in the pictures they show as examples for elongated centrioles, they rather seem to have supernumerary centrioles than long ones. The elongated centrioles shown in figures 4 C and E look like three distinct centrioles. The quality of the images is also low and perhaps makes it more difficult to appreciate whether there are longer centrioles present. Perhaps using a proximal and a distal marker would make it easier to quantitate, or centrin intensity, as we have suggested before.*

2. We provide a new Fig. 4F, G, and H, where we measure elongated centrioles using centrin antibody. The elongated centriole appears as a thread that can clearly be distinguished from supernumerary centrioles (see inset in Fig. 4F right panel). To measure the frequency of elongated centrioles we have performed three independent experiments (Fig. 4F and G) and obtained similar results as with anti-CP110 (shown in Fig. 4C and D). For measurement of the length we measured 40 centrioles (Fig. 4H) and obtained similar results as with anti-CP110 (shown in Fig. 4E).

*Regarding figure 4H, it would be better to score numbers with two centriolar markers, instead of just one, because centrin can also form aggregates.*

The point of Fig. 4H (now 4K) was to show multipolarity. Hence, we co-stained with centrin and alpha tubulin in order to score the pole and the nucleating microtubues surrounding it. The observation that the spindle is generated at each centrin spot indicates that it is not aggregation but rather that a spindle is being generated.

*This data would also gain tremendously if the authors could express their CPAP-AA mutant that does not bind tankyrase, and compare its cellular phenotype to the expression of CPAP WT version. This would indeed be a more direct proof that tankyrase regulates directly CPAP levels and centriole function.*

The focus of our manuscript is to show the impact of tankyrase 1 on CPAP expression and function. We feel that our demonstration that knockdown of tankyrase 1 regulates stabilization of CPAP precisely in the narrow window of the

cell cycle where it is normally degraded, combined with all the other in vitro and in vivo data, make a striking case for a role for tankyrase 1 in CPAP function.

*Figure 5-FACS profile could not be seen in my manuscript version.*

We have fixed the FACS profile.

3rd Editorial Decision 23 May 2012

Thank you for the submission of your revised manuscript to our offices. I think it fully addresses all remaining concerns and I am thus happy to accept it for publication in EMBO reports. However, I have noticed that the manuscript does not contain a materials and methods section in the main text. Basic materials and methods essential to the understanding of the experiments must be described in the main body of the manuscript, although more detailed explanations necessary to reproduce them may be presented as supplementary information. As the total manuscript length is already at its maximum, this means that you will need to go through the text one more time to make it more succinct, ensuring you have space for the methods section.

Please send us your new, final text file and supplementary information file as email attachments and we will upload them to our system.

Many thanks for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports