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Efficient mitotic checkpoint signaling depends on integrated activities of Bub1 and the RZZ complex.

Gang Zhang, Thomas Kruse, Claudia Guasch Boldú, Dimitriya H. Garvanska, Fabian Coscia, Matthias Mann, Marin Barisic& Jakob Nilsson.

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Transaction Report:

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1st Editorial Decision 20th November 2018

Thank you again for submitting your manuscript on Mad1 receptor and SAC roles of Bub1 and RZZ for our editorial consideration. I have now heard back from two expert referees, whose reports are copied below for your information. With both referees acknowledging the potential importance and overall quality of this work, we shall be happy to consider a revised version further for expedited publication in The EMBO Journal. As you will see, the reviewers raise a limited number of specific concerns, most of which related to presentation, controls and statistical analyses, which I hope should be straightforward to address. With regard to referee 2's major point, 1do not feel that all the various avenues suggested there necessarily need to be addressed with further experimentation for the scope of this revision, but would nevertheless encourage you to carefully consider these points and follow up at least on some of the more concrete/control-relevant issues noted here by reviewer 2.

REFEREE REPORTS

Referee #1:

In this manuscript, Zhang et al study the contribution of two kinetochore components, Bub1 and the RZZ complex, to the signaling of the spindle assembly checkpoint (SAC). This work tries to clarify a confusing situation with regard to the role of Bub1 in the SAC signaling. Previous work had shown that Bub1 is essential for the SAC in yeast (Hoyt et al., 1991, Vanoosthuyse et al., 2004), Drosophila (Basu et al., 1999) and mice (Perera et al., 2007), but two recent studies based on stable CRISPR/Cas9 depletions (Currie et al., 2018; Raaijmakers et al., 2018) reported that Bub1 is not essential for the SAC in humanc cells. This contradicted previous studies based on siRNAs that had found an essential role for Bub1 in the checkpoint (Meraldi and Sorger, 2005; Klebig et al., 2009, but also more recent Zhang et al., 2017). However, a very recent study from the Jallepalli laboratory

(Rodriguez-Rodriguez et al., 2018) reported that the presence of a checkpoint in Bub1 CRISPR/KO cells could be due to the low expression of an alternatively spliced Bub1.

This present study aimed to clarify this situation and the authors convincingly show by mass-spectroscopy that their own Bub1 KO cells as well as the previously described Bub1 KO still express low levels of Bub1. Subsequent depletion of this residual Bub1 population greatly diminishes the SAC response, arguing that human Bub1 has a conserved role in SAC signaling. In addition, the authors study the role of the RZZ complex using the same combination of CRISPR/Cas9 KO, siRNA as well as expression of different recombinant protein. Based on their results that show that the RZZ participates to the SAC signaling by recruiting Mad1 to kinetochores. This role is complementary to Bub1, which contributes to the SAC signaling through two mechanisms: the binding of Mad1 to kinetochores, as well as another yet-to-define mechanisms that cannot be rescued by forced recruitment of Mad1 to kinetochores.

Overall, this is an important study that will greatly clarify the respective role of Bub1 and RZZ in the checkpoint signaling, which is strongly needed at present in the field. Although a very recent paper from the Jallepalli laboratory (Rodriguez-Rodriguez et al., 2018) comes to the same conclusions, this present study is complementary and goes further in particular in showing by mass-spectroscopy that the CRISPR/Cas 9 cell lines from the McAinsh/Millar and Medema laboratories still expresses residual Bub1 protein. There are a number of technical and minor interpretation concerns that should be addressed before publication (see below), but once addressed, I strongly support publication of this study in EMBO Journal.

Major points:
1) The authors demonstrate by mass-spectroscopy that the CRISPR/Cas9 Bub1 KO cells still express residual levels of Bub1. First, the authors only show this for their own KO cells in the main figure, while relegating the analysis of the published RPE1 and HAP1 cells into the supplementary figures. If possible, they should show the analysis of all cell lines in the main figure, as this is a central point of the paper. Second, for consistency it would be important to demonstrate by mass-spectroscopy that Bub1 KO + Bub1 siRNA (called Bub1 CR) further diminishes Bub1 levels. This would also show by how much Bub1 needs to be depleted to inactivate the SAC.

2) The authors indicated for all the mitotic timing experiments only the number of cells, not the number of independent experiments. Moreover they do not provide statistical analysis to demonstrate that the reported differences are significant (I assume they are, since the differences are large). This information has to be provided to the reader.

3) While the authors make a strong point that Bub1 knockdown/knock-out severely impairs SAC signaling, it might be important to state more explicitly that this does not correspond to a complete abrogation of the checkpoint, as seen after depletion of Mad2 or BubR1, as Bub1 CR cells still remain for 110 min in mitosis in the presence of nocodazole. Only when Rod is also depleted do the authors see a very rapid mitotic exit. This suggests that in the complete absence of Bub1, the RZZ complex can still prolong mitosis duration 3-fold, which one could interpret as an indication that its only role is not to only bring Mad1 in the vicinity of Bub1. The authors might therefore consider discussing these points in a more explicit, yet less absolute manner.

Patrick Meraldi

Referee #2:

The SAC is locally activated on unattached kinetochores to generate the MCC complex (formed by BubR1/Bub3/Cdc20/Mad2). The essential players of the SAC are known and the catalytic process that leads to MCC assembly has recently been reconstituted in vitro: Bub1 and Mad1 play a catalytic role in the assembly of the MCC by bringing the components of the MCC into close proximity. However, there are major discrepancies in the field on how this process takes place at the kinetochore, especially on the relative contribution of Bub1 and RZZ in localizing Mad1. Furthermore, recent papers using Bub1 KOs have questioned the importance of Bub1 in MCC assembly in cells. The authors dedicate great effort to clarify this discrepancy and show that

previous conclusions were based on incomplete depletions of endogenous proteins, even in CRISPR KO backgrounds. By combining partial CRISPR-KO mutants with siRNA treatment, the authors achieve highly penetrant depletions of Bub1 and the RZZ complex. This allows them to demonstrate that:
1. Bub1 plays a major role in catalyzing MCC assembly.
2. RZZ lacks a catalytic role in MCC assembly.
3. Although both BUB1 and RZZ contribute to the localization of MAD1, RZZ is responsible for keeping Mad1 stable on the kinetochore.

These conclusions agree and somewhat overlap with a recent paper from the Jallepalli lab (Rodriguez-Rodriguez et al., Curr Biol 2018). Both works also put a focus on the necessity of performing additional controls and depletion strategies when knocking out essential genes. Zhang et al. provide additional molecular details on the domains of BUB1 that contribute for the recruitment of MAD1 and RZZ.

I overall support publication of this manuscript, provided the following issues are addressed:

1- Although the authors do a good job in clarifying previous discrepancies, their study provides few mechanistic insights on the roles of Bub1 and RZZ in the SAC. The manuscript would gain relevance if the biological significance of having two integrated but independent pathways of MAD1 recruitment would be addressed. The authors rescue the localization of MAD1 in the ROD CR condition by expressing a mutant of Bub1 with predicted higher Mad1 binding capacity (Bub1 4xCD1), but they stop after showing it can rescue the SAC in noco cells. Various controls are missing and there is an opportunity here to make this paper more impactful:
- Whether or not 4xCD1 has higher binding is not shown: does 4xCD1 localize more Mad1 than WT in a BUB1si-reconstitution experiment?
Can stable expression of Bub1 4xCD1 allows to obtain full ROD1 KOs. That would be a proof that the function of RZZ can be bypassed by recruiting enough MAD1.
- What are the consequences of expressing this mutant in unperturbed mitosis?: Characterize normal mitosis progression, alignment and missegregations in cells expressing BUB1 4xCD in the presence and absence of ROD. Assess strength of the SAC in taxol in the same backgrounds. Related with this experiment, I find interesting that ROD CR shows a stronger checkpoint in taxol (160') than in nocodazole (90'), where more unattached kinetochores are expected to be present. I wonder if the authors have any theory about it as it might actually reflect differences in how RZZ and BUB1 respond to attachment defects. Furthermore, I could not find the concentration of taxol used.
- By analyzing MAD1 levels at different time points in nocodazole arrested cells, Rodriguez-Rodriguez et al. have shown that RZZ is required to keep MAD1 stable on kinetochores. The authors only show MAD1 levels after 45 minutes in nocodazole arrest but, for instance, ROD CR is able to maintain the checkpoint on average 90'. I suggest to do time series of Mad1 kinetochore levels in nocodazole in the BUB1 CR and ROD CR background. Also, include in the analysis BUB1 4xCD1 to compare. I think this control is important as it might reflect differences in the way RZZ and BUB1 respond to phosphatases after prolonged mitosis arrest.
- A suggestion (not an issue to address necessarily): Two pools of MAD1 on unattached kinetochores has been described before (Defachelles L. et al. 2015, Sha et al. 2004, Howell et al. 2004): a dynamic one and a more stable one. To my knowledge, the origin of these two pools have not been formally addressed, but it is tempting to speculate they might correspond to Bub1 and RZZ. Furthermore, previous work has also suggested that Bub1 accelerates Mad1 loading (Vleugel et al., 2015). Since the experiments in Figure 4F show that the absence of ROD does not affect Mad1 dynamics, it would be informative to check if in the absence of BUB1 the RZZ-dependent pool of Mad1 is completely stable or if the two pools of Mad1 result from an interplay between Bub1 and RZZ. To address this, the authors might consider to include FRAP experiments of Mad1 in Bub1 CR in nocodazole treated cells, especially after prolonged mitotic arrest when a fibrous corona is present and relatively high levels of Mad1 are observable, according to Rodriguez-Rodriguez et al.

2- By combining in their analysis previously published Bub1 KO cell lines with siRNA treatments, the authors make a good point to disprove that Bub1 has a marginal role in SAC activation. This is in line with recent in vitro studies showing that Bub1 has a catalytic role in MCC assembly (Musacchio lab, Faessen et al Nature 2017). However, BUB1CR cells still show a significant arrest (compared to the combined depletion of Bub1 and ROD), and BUB1C actually shows a normal

checkpoint. Rodriguez-Rodriguez et al. report that Bub1 KOs frequently result in (at least partially) functional protein via alternative splicing. Thus, it is important to perform RT-PCRs in the two BUB1 C clones used, and address if any Bub1 peptide can still be detected in the Bub1CR conditions, assayed like in Figure 3C. This is important to remove any lingering discussion on Bub1's role in the SAC (the key objective of this study) and interpret the origin of the delay present in BUB1CR and examine if really no delay can be mounted when Bub1 is absent.

3- In general, the number of cells used in the analyses is too low: Around 10 cells for the immunofluorescence studies and 20-40 in the live experiments. Also, it is often not stated how many independent repeats were performed. In our experience, it is difficult to make robust conclusions from such low numbers of cells.

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2. Legend of Figure S2 does not properly refer to the different panels.
3. In table 1, where is written Roc CR should be Rod CR.
4. In page 6, where it refers to the mutant of Rod that lacks the β-propeller domain, the corresponding reference should be included.

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Our response:

We have performed this analysis and as expected we see are strong reduction in Bub1 peptides in our Bub1 CR cell lines compared to Bub1 C. We only detected 3 low intensity Bub1 peptides in the mass spectrometry runs of Bub1 CR. This shows that Bub1 RNAi in Bub1 C cells further reduces Bub1 levels as we predicted. Based on the low number of peptides we cannot accurately estimate the level of Bub1 in Bub1 CR. We have also analyzed RPE1 WT vs RPE1 Bub1 KO cells and HAP1 WT vs HAP1 Bub1 KO cells now and included this in the main figure (new figure 3C-D). Furthermore we performed mock purifications (beads run in parallel with purifications and just treated with buffers) to ensure no cross contamination was occurring. Analysis of these purifications revealed only 1 low intensity Bub1 peptides arguing that the peptides we are detecting are not due to contamination between samples.

We want to point out that we had swapped the labels in the Bub1 RNAi experiments with RPE1 Bub1 KO and HAP1 Bub1 KO in the original submission and this has now been corrected (Expanded view figure 2A).

2) The authors indicated for all the mitotic timing experiments only the number of cells, not the number of independent experiments. Moreover they do not provide statistical analysis to demonstrate that the reported differences are significant (I assume they are, since the differences are large). This information has to be provided to the reader.

Our response:

We agree with the reviewer and have now added this information to the figures and figure legends as well as the materials and methods. All time-lapse experiments have been performed at least 3 times and we show a representative experiment. All immunofluorescence analysis have been performed at least 2 times with 200 kinetochores quantified from 10 individual cells per experiment. As an example of consistency compare Mad1 levels in siRod in figure 1E, figure 1g and figure 2B - these experiments are performed at very different times but give similar results. Similar one can compare Bub1C and Bub1 CR time-lapse results in figure 3A and 6A, Rod C and Rod CR in figure 2D and 5C that are very consistent but have been performed at very different times.

3) While the authors make a strong point that Bub1 knockdown/knock-out severely impairs SAC signaling, it might be important to state more explicitly that this does not correspond to a complete abrogation of the checkpoint, as seen after depletion of Mad2 or BubR1, as Bub1 CR cells still remain for 110 min in mitosis in the presence of nocodazole. Only when Rod is also depleted do the authors see a very rapid mitotic exit. This suggests that in the complete absence of Bub1, the RZZ complex can still prolong mitosis duration 3-fold, which one could interpret as an indication that its only role is not to only bring Mad1 in the vicinity of Bub1. The authors might therefore consider discussing these points in a more explicit, yet less absolute manner.

Our response:

The reviewer is absolutely correct in that RZZ contributes to SAC signaling and can do so in the absence of Bub1. We assume this is through its role in localizing Mad1 although difficult to test as we do not have an "RZZ" mutant that is specifically defective in Mad1 localization. We have pointed out this contribution of RZZ to the SAC more carefully in the revised manuscript:

"The Mad1 tethering experiments support that the main function of the RZZ complex is to localize Mad1 to kinetochores. Although the RZZ complex is less efficient in inducing a SAC arrest in the absence of Bub1 it is capable of delaying mitosis approximately 3-fold likely through RZZ mediated Mad1 localization. This is consistent with our mass spectrometry analysis of BubR1 purifications from mitotic Bub1 CR cells that reveal similar MCC levels as controls. Why RZZ in the absence of Bub1 cannot maintain a prolonged SAC arrest despite MCC generation is unclear to us but could possible reflect a change in SAC silencing activities with time."
"

Referee #2:

The SAC is locally activated on unattached kinetochores to generate the MCC complex (formed by BubR1/Bub3/Cdc20/Mad2). The essential players of the SAC are known and the catalytic process that leads to MCC assembly has recently been reconstituted in vitro: Bub1 and Mad1 play a catalytic role in the assembly of the MCC by bringing the components of the MCC into close proximity. However, there are major discrepancies in the field on how this process takes place at the kinetochore, especially on the relative contribution of Bub1 and RZZ in localizing Mad1. Furthermore, recent papers using Bub1 KOs

have questioned the importance of Bub1 in MCC assembly in cells. The authors dedicate great effort to clarify this discrepancy and show that previous conclusions were based on incomplete depletions of endogenous proteins, even in CRISPR KO backgrounds. By combining partial CRISPR-KO mutants with siRNA treatment, the authors achieve highly penetrant depletions of Bub1 and the RZZ complex. This allows them to demonstrate that:
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I overall support publication of this manuscript, provided the following issues are addressed:

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- Whether or not 4xCD1 has higher binding is not shown: does 4xCD1 localize more Mad1 than WT in a BUB1si-reconstitution experiment?
Can stable expression of Bub1 4xCD1 allows to obtain full ROD1 KOs. That would be a proof that the function of RZZ can be bypassed by recruiting enough MAD1.

Our response:

We thank the reviewer for these suggestions. We have in the revised manuscript analyzed the Bub1 4xCD1 mutant more extensively. Firstly we now show that it can support checkpoint signaling and efficiently recruits Mad1 to kinetochores in cells lacking Bub1 (Bub1 CR, new figure 6). The level of Mad1 recruited in Bub1 4xCD1 is substantially higher than in Bub1 1XCD1 complemented cells yet the strength of the checkpoint is not significantly different. There must therefore be

additional parameters that set the limits to SAC strength beyond Bub1-Mad1 interaction, which we point out. It should be noted that our Bub1 4xCD1 construct does not contain the ABBA motifs required for Cdc20 interaction that might be required for full checkpoint activation.

We find that analysing if Bub1 4xCD1 stable expression allows generation of full Rod CRISPR KO is beyond the scope of the current study, as this would require a massive endeavor. Furthermore since Rod has clear functions in chromosome segregation independent of its role in Mad1 recruitment it is likely that the inability to generate Rod KO is due to this function of Rod.

- What are the consequences of expressing this mutant in unperturbed mitosis?: Characterize normal mitosis progression, alignment and missegregations in cells expressing BUB1 4xCD in the presence and absence of ROD.

Our response:

We have now analyzed the effect of Bub1 4xCD1 on unperturbed mitosis and alignment (new Figure 6). There are no dominant effects detected when Bub1 4xCD1 is expressed. As multiple SAC silencing mechanisms exists (for example PP1 dephosphorylation of MELT repeats that would remove Bub1 4xCD1 from kinetochores) these mechanisms might be sufficient to silence the SAC upon microtubule attachment. The lack of a dominant negative effect from Bub1 4xCD1 is also consistent with the SAC strength being similar to Bub1 1-529 that only has a single CD1 (see point above). As there is no dominant effect from Bub1 4xCD1 we did not analyze it in Rod CR.

Assess strength of the SAC in taxol in the same backgrounds.

Our response:

We find that the test in nocodazole is sufficient to make the point of the paper.

Related with this experiment, I find interesting that ROD CR shows a stronger checkpoint in taxol (160') than in nocodazole (90'), where more unattached kinetochores are expected to be present. I wonder if the authors have any theory about it as it might actually reflect differences in how RZZ and BUB1 respond to attachment defects. Furthermore, I could not find the concentration of taxol used.

Our response:

We find this difference interesting as well but at present we do not have a good explanation and have not explored this sufficiently to warrant any conclusions. We have added taxol concentration.

- By analyzing MAD1 levels at different time points in nocodazole arrested cells, Rodriguez-Rodriguez et al. have shown that RZZ is required to keep MAD1 stable on kinetochores. The authors only show MAD1 levels after 45 minutes in nocodazole arrest but, for instance, ROD CR is able to maintain the checkpoint on average 90'. I suggest to do time series of Mad1 kinetochore levels in nocodazole in the BUB1 CR and ROD CR background. Also, include in the analysis BUB1 4xCD1 to compare. I think this control is important as it might reflect differences in the way RZZ and BUB1 respond to phosphatases after prolonged mitosis arrest.

Our response:

Our live cell analysis of Mad1-Venus localization is fairly consistent with the observations in the Rodriguez-Rodriguez paper and also as reported by the Kops lab earlier using Bub1 RNAi (Vleugel et al, JCS 2015) and by the Bollen lab (Qian J, Mol Cell 2017). By live cell in both Bub1 CR and Rod CR we see reduced Mad1 kinetochore levels consistent with our IF analysis in Figure 1. In Rod CR we see that the Mad1-Venus kinetochore signal disappears after some time and cells exit consistent with Mad1 localization becoming more dependent on RZZ with time. We discuss this extensively in the manuscript discussion and how modulation of Bub1 phosphorylation status might affect this shift.
Given that our live cell analysis already addresses this point and that several papers have explored this we do not find that this would add to the paper. It is also important to point out that the penetrant removal of SAC proteins is mainly needed to detect phenotypes while the effect on SAC protein localization can fairly well be analyzed with robust RNAi oligoes (this point is illustrated in Figure 2B and also mentioned in the text).

- A suggestion (not an issue to address necessarily): Two pools of MAD1 on unattached kinetochores has been described before (Defachelles L. et al. 2015, Sha et al. 2004, Howell et al. 2004): a dynamic one and a more stable one. To my knowledge, the origin of these two pools have not been formally addressed, but it is tempting to speculate they might correspond to Bub1 and RZZ. Furthermore, previous work has also suggested that Bub1 accelerates Mad1 loading (Vleugel et al., 2015). Since the experiments in Figure 4F show that the absence of ROD does not affect Mad1 dynamics, it would be informative to check if in the absence of BUB1 the RZZ-dependent pool of Mad1 is completely stable or if the two pools of Mad1 result from an interplay between Bub1 and RZZ. To address this, the authors might consider to include FRAP experiments of Mad1 in Bub1 CR in nocodazole treated cells, especially after prolonged mitotic arrest when a fibrous corona is present and relatively high levels of Mad1 are observable, according to Rodriguez-Rodriguez et al.

Our response:

We have performed FRAP analysis of Mad1-Venus in Bub1 CR now (Supplementary Figure 4). The turnover of Mad1-Venus is not affected by removal of Bub1 suggesting that the interaction with RZZ is not stable in agreement with the inability to detect a robust biochemical interaction between these proteins by the field. Note that we had to use slightly different settings and Mad1-Venus to perform the FRAP to get better data which resulted in a slightly different half-life of Mad1 in the control condition. However this does not affect the overall conclusion that Mad1 interacts dynamically with both Bub1 (Rod CR FRAP) and RZZ (Bub1 CR FRAP). One important point is that in Bub1 CR the levels of RZZ might also be affected.

2- By combining in their analysis previously published Bub1 KO cell lines with siRNA treatments, the authors make a good point to disprove that Bub1 has a marginal role in SAC activation. This is in line with recent in vitro studies showing that Bub1 has a catalytic role in MCC assembly (Musacchio lab, Faessen et al Nature 2017). However, BUB1CR cells still show a significant arrest (compared to the combined depletion of Bub1 and ROD), and BUB1C actually shows a normal checkpoint. Rodriguez-Rodriguez et al. report that Bub1 KOs frequently result in (at least partially) functional protein via alternative splicing. Thus, it is important to perform RT-PCRs in the two BUB1 C clones used, and address if any Bub1 peptide can still be detected in the Bub1CR conditions, assayed like in Figure 3C. This is important to remove any lingering discussion on Bub1's role in the SAC (the key objective of this study) and interpret the origin of the delay present in BUB1CR and examine if really no delay can be mounted when Bub1 is absent.

Our response:

We thank the reviewer for these comments. See also above comments to reviewer 1 suggestion on analyzing Bub1 CR by MS. When we map the peptides we detect in HeLa Bub1 C and RPE1 and HAP1 Bub1 KO we do not detect any peptides in the N-terminal part of the proteins where the gRNAs used are targeting Bub1 (new expanded view figure 3). This could suggest that this part of Bub1 is missing in these cell lines – importantly this part of Bub1 is likely not required for the SAC. We have not performed RT-PCR analysis as we favor to have experimental evidence at the protein level, as transcripts might not give rise to protein. The important point is that there is Bub1 protein in reported Bub1 KO cell lines.

3- In general, the number of cells used in the analyses is too low: Around 10 cells for the immunofluorescence studies and 20-40 in the live experiments. Also, it is often not stated how many independent repeats were performed. In our

experience, it is difficult to make robust conclusions from such low numbers of cells.

Our response:

We have indicated this now in figures and figure legends and included relevant statistical tests. It is important to note that our clean genetic background gives highly significant results while previous Bub1 RNAi studies are difficult to interpret since they are not true null experiments. All conclusions are based on highly statistical significant results. See also our comment to point 2 or reviewer 1.

Minor issues:
1. Indicate in the legend of Figure 2E what the red circles represent. In Figure 3E the red circles represent cells that did not exit during the duration of the film. Does that mean they die during the duration of the experiment? Then I consider they should not be included in the analysis. In figure 5B, red circles can either mean that cells stayed in mitosis at the end of the recording or die, I would suggest remove the cells that die.

Our response:

We have indicated this more precisely now in the legends. Red circles indicates cells that are still arrested in a mitotic state at the end of the recording. This is not dead cells that we do not include in the analysis. In general we only detect few dead cells.

2. Legend of Figure S2 does not properly refer to the different panels.

Our response:

Improved legend in revised manuscript.

3. In table 1, where is written Roc CR should be Rod CR.

Our response:

Corrected.

4. In page 6, where it refers to the mutant of Rod that lacks the b-propeller domain, the corresponding reference should be included.

Our response:

We have now included a reference.

2nd Editorial Decision 22nd January 2019

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by the two original reviewers, whose comments are copied below. Both of them are generally satisfied with the revisions, with only referee 2 retaining a few specific reservations. We shall therefore be happy to accept this work for The EMBO Journal, following minor text modifications in response to particularly the second point of referee 2.

REFEREE REPORTS

Referee #1:

The authors have addressed all my concerns. They have in particular shown that in the Bub1 KO cells treated with a Bub1 siRNA there are no detectable Bub1 peptides any more. Since the authors also have addressed most concerns of reviewer 2, I fully support publication of this important study.

Referee #2:

The authors have addressed and clarified most of the concerns raised. I would like to reinforce that although this paper explains recent discrepancies in the field, in my view, it does not provide novel insights on the role of RZZ and Bub1 in SAC activation. And that is what I would like to have seen in the revised version, especially when I suggested to further analyze the mutant Bub1-4xCD1 in the absence of Rod. I found this mutant interesting because in contrast to Knl1/Hec1-Mad1 fusions (see Rodriguez-Rodriguez et al.), this mutant still depends on native Mps1 phosphorylations to recruit Mad1. It is therefore not surprising that it does not dominantly sustain a checkpoint, and therefore interesting to explore how it performs in the absence of RZZ. My argument to do this experiment was that it might reveal the importance of the second Mad1 receptor when phosphatases impinge. Secondly, I am still not completely convinced that the delay observed in Bub1CR is independent of residual Bub1 or if indeed RZZ is able to activate to some extent the checkpoint in the absence of Bub1. I have no experience with sensitivity of qPCR vs MS, but with the evidence shown (few peptides detected in Bub1CD) it cannot be discarded that the observed 3-fold delay is still Bub1 dependent. Thus, I think this scenario should be explicitly mentioned in the discussion (page 14).

2nd Revision - authors' response 23rd January 2019

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peptides detected in Bub1CD) it cannot be discarded that the observed 3-fold delay is still Bub1 dependent. Thus, I think this scenario should be explicitly mentioned in the discussion (page 14).

Our response:

We have now included the following sentence in our discussion:

At present we cannot rule out that undetectable amounts of Bub1 remaining in Bub1 CR are responsible for the delay seen in these cells. However we do not favor this because all the mitotic timings are tightly clustered around 110 minutes in nocodazole which is very similar to that obtained when dominant negative versions of Bub1 are expressed (Zhang et al, 2017).

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Jakob Nilsson
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2018-100977

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical test was used to predetermine sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All mitotic cells from time lapse experiments were analyzed. We only excluded experiments where we could see that the controls did not work.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes. We use a Mann Whitney test for time-lapse data as mitotic timings are not normal distributed and this is therefore the appropriate test to use.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. For time-lapse data we use a Mann Whitney test as the data is not normal distributed. We only performed statistical analysis of immunofluorescence in figure 7F and here we used a t-test as the kinetochore levels of a checkpoint protein is normal distributed. All conclusions drawn from immunofluorescence experiments are based on clear and obvious effects and we have therefore not performed further statistical analysis.
Is there an estimate of variation within each group of data?	No
Is the variance similar between the groups that are being statistically compared?	No

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We have provided either reference or catalog numbers for antibodies. Home made antibodies have been used for a number of blots and are all extensively characterized and described in the references cited.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HeLa cells are from ATCC and original cell lines were tested for mycoplasma and stock made from these. We have not recently authenticated this cell line. RPE1 cells used in Figure 1 were obtained from Jonathan Pines and we have not authenticated these. RPE1 wt and RPE1 Bub1 KO was provided from Jonathan Millar and we have not authenticated these. HAP1 wt and HAP1 Bub1 KO was from Rene Medema and we have not authenticated these. U2OS cells was obtained from Niels Mailand lab. We have not tested for mycoplasma except for the original stocks of HeLa cells.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The mass spectrometry data will we made available through PRIDE
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.